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Inhibitor studies of root water flow regulation in aspen (*Populus tremuloides* Michx.) seedlings

by

Mihaela Cristina Voicu

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

in

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Abstract

Water movement across cell membranes is largely facilitated by aquaporins (water channel proteins). The present study investigated mechanisms of root water flow regulation through phosphorylation/dephosphorylation processes and protein synthesis. Roots of solution culture-grown aspen (*Populus tremuloides* Michx.) seedlings were treated with different chemical inhibitors of the above processes and the steady-state root water flow, root respiration, acid phosphatase activity, stomatal conductance, root hydraulic conductivity and apoplastic water flow were examined. Most of the phosphorylation/dephosphorylation inhibitors reduced the root water uptake and the activity of acid phosphatases while they had no effect on root respiration. The protein synthesis inhibitor rapidly inhibited root water flux and aquaporin expression suggesting that transcriptional and (or) translational regulation of aquaporin synthesis could play a significant role in regulating root water uptake. The phosphorylation/dephosphorylation events and protein synthesis appear to be among the primary processes regulating root water uptake.

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List of Symbols and Abbreviations

σ – reflection coefficient for solutes.
$\Delta\pi$ – osmotic force
ΔP – pressure difference or hydrostatic pressure
6-DMAP – 6-dimethyl aminopurine
A – root surface area
ABA – abscisic acid
ADP – adenosine diphsophate
ATP – adenosine triphosphate
ATPases – ATP hydrolyzing enzymes
BCA – bicinchoninic acid
BCIP – 5-bromo-4-chloro-3-3indolyl phosphate
CAM – Crassulacean acid metabolism
CDPK – calcium dependent calmodulin-independent protein kinase
CHX – cycloheximide
CPU – central processor unit of a computer
DMF – dimethy-formamide
dP – pressure gradient
DTT – dithiotreithol
dx – length
E_a – Arrhenius activation energy
ER – endoplasmic reticulum
F – water flow rate
GLM – general linear model
g_s – Stomatal conductance
H-7 – 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine
J_v – water flux density
K – absolute hydraulic conductance
k_h – absolute hydraulic conductivity
L_p – root hydraulic conductivity
MAPK – mitogen activated protein kinases
MIPs – major intrinsic proteins
NBT – nitro blue tetrazolium
NPA – asparagine-proline-alanine
OA – okadaic acid
P_d – coefficient of diffusional permeability
Pi – inorganic phosphate
PIPs – plasma membrane intrinsic proteins
PKA – cAMP-dependent protein kinase
PM – plasma membrane
P-NPP – p-nitrophenyl phosphate
P_{os} – coefficient of osmotic water permeability
PPM – protein phsophatase M
PPP – protein phsophatase P

PP1 – protein phophatase-1
PP2A – protein phophatase-2A
PP2B – protein phosphatases-2B
PP2C – protein phoshphatase-2C
PTK – protein tyrosine kinase
PTPase – protein tyrosine phosphatases
PTS_3 – trisodium 3-hydroxy-5,8,10-pyrenetrisulphonate
PVP-40 – polyvinvyl pyrolidone
PVPP – polyvinylpolypyrrolidone
Q_v – steady-state root water flow
RLKs – receptor-like kinases
SDS – sodium dodecyl sulfate
SDS PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser/Thr – serine and threonine
SIP – small intrinsic proteins
SPAC – soil-plant-atmosphere continuum
TBS – Tri-buffered saline solution
TIPs – tonoplast intrinsic proteins
W-7 – N-(6-Aminohexyl)-5-chloro-1-naphtalene-sulfoamide
Ψ_w – leaf water potential

Chapter 1. General introduction and literature review

1.1. Introduction

Water plays a crucial role in various physiological processes in plants (Lambers et al. 1998). Water is used as a medium for all resource exchanges between a plant and its environment (Nilsen and Orcutt 1996). Plants use water in biochemical reactions, as a solvent and to maintain turgor, but most of the water taken up by plants (more than 99%) is transpired to the atmosphere (Jackson et al. 2000).

In higher plants, water enters the plant through its roots. Root water uptake and transport represent the gateway for plant water supply (Jackson et al. 2000). Roots can modulate the process of water absorption in response to different environmental stimuli. The reasons for the variability in root water uptake are still little understood. The discovery of aquaporins (proteins that form water channels in the cell membranes) has shed new light on the regulation of water fluxes within plant tissues. At the whole plant level, the picture is more complicated since water balance of the plant depends on the capacity of roots to absorb water and on the rate of water loss through transpiration.

In order to understand the modulation of water fluxes across the root, we need to understand how this process is regulated at the membrane, cell, tissue, and organ levels.

1.2. Driving forces for water movement in plants

The movement of water through the soil-plant-atmosphere continuum (SPAC) has been considered analogous to the flow of electricity in a conducting system (Tyree and Ewers 1991). Water moves passively in response to water potential gradients (Steudle 2001). In transpiring plants, water loss from the stomatal chamber results in highly negative pressures in tracheary elements, which draw water from roots into aerial parts (Steudle 2001, Javot and Maurel 2002). In the absence of transpiration, when stomata are closed, ion uptake from the soil creates an osmotic driving force that pulls the water from soil and pushes it through the xylem (Steudle and Peterson 1998, Javot and Maurel 2002). However, water movement into the root is driven by both hydraulic and osmotic forces (Steudle and Peterson 1998).

1.3. Root anatomy

The physical properties of roots are related to their anatomy (Steudle and Peterson 1998). To reach the xylem inside the roots, water has to pass through several layers of cells. Typically, these are the epidermis, cortex, endodermis, and pericycle.

Cells of the endodermal layers possess radial and transverse thickenings of the cell walls called Casparian bands. The main component of the Casparian band is suberin that is impermeable to water. However, some studies suggest that the endodermis does not always form a permanent impermeable barrier to water and solutes (Peterson et al. 1993, Steudle et al. 1993).

Roots of the soil-grown plants often develop an exodermis. This cell layer inside the epidermis also develops Casparian strips that typically have a suberin lamella (Peterson et al. 1993).

1.4. Pathways of water movement into roots

1.4.1. Composite model

Roots transport water from soil to shoots. This transport may occur through different pathways: a) apoplastic pathway through the intercellular spaces, cell walls, and tracheary elements, b) symplastic pathway through the cytosol and plasmodesmata, and c) transcellular pathway that involves water movement across the plasma membranes and the tonoplasts (Carvajal et al. 1998, Steudle 2000b) (Figure 1.1). The transcellular and symplastic paths cannot be measured separately and, therefore, they are referred to as the "cell-to-cell" pathway (Steudle and Peterson 1998, Steudle 2000b).

Relative contributions of the apoplastic and cell-to-cell pathways can vary, making it difficult to quantify water flow through the different pathways (Tyreman et al. 1999). The three pathways also interact with each other resulting in measurable phenomena such as the circulation or flow of water and a low overall reflection coefficient of the root (Steudle 2000b).

Water transport in the apoplast is driven by hydrostatic forces, while for the cell-to cell pathway, osmotic gradients in addition to hydrostatic gradients drive the water flow (Steudle and Peterson 1998).

1.4.2. Apoplastic pathway

The apoplast includes the cell walls, intercellular spaces and the lumena of tracheary elements. Since the apoplastic pathway does not discriminate between water and solutes, the reflection coefficient is close to zero (Steudle 2000b). The reflection coefficient denotes passive selectivity of a membrane or a barrier, and has a value between zero (osmotic barrier has no selective properties) and unity (solutes are completely retained by the barrier) (Steudle and Peterson 1998).

Changes in the apoplast include development of the endodermis, exodermis, and aerenchyma (in the case of flooding stress). The exodermis and endodermis are relatively impermeable to water, posing a barrier to water movement (Steudle and French 1996, Zimmermann and Steudle 1998). Anatomical changes provide only a slowly reversible (growth-dependent) or irreversible adjustment to root hydraulic conductivity (Tyreman et al. 2002).

1.4.3. Symplastic pathway

The symplastic movement of water takes place through the plasmodesmata, which create a cytoplasmic continuum among cells (Carvajal et al. 1998).

Relatively few studies have focused on the involvement of plasmodesmata in water transport. Zhang and Tyreman (1991) explained the decrease in hydraulic conductivity of oxygen-deprived root cortical cells by the occlusion of plasmodesmata. Using the pressure-clamp technique, Murphy and Smith (1998), concluded that symplastic water

transport contributes significantly to water transport of cells, especially at low pressure gradients (about 0.08-0.16 MPa). There is evidence that the solute transport can also occur via desmotubules (Cantrill et al. 1999), while the organelles such as the endoplasmatic reticulum may also have water channels (Kikuyama and Tazawa 1998).

Because at pressure differentials equal or greater than 0.2 MPa plasmodesmata close, Fricke (2000) suggested that the symplastic water flow contributes insignificantly to the water exchange between cells.

In whole plants, all three pathways (apoplastic, symplastic, and trancellular) contribute to the water transport. Therefore, different chemical inhibitors that inhibit water transport in roots could also inhibit water flow through the plasmodesmata (Tazawa et al. 2001).

1.4.4. Transcellular pathway

Water may be transported across the plasma membrane by two fundamentally distinct pathways: 1) simple diffusion through the lipid bilayer; and 2) channel-mediated transport, facilitated by water channel proteins (Finkelstein 1987, Agre et al. 1993, Schäffner 1998).

1.4.4.1. Diffusional water permeability

The coefficient of diffusional permeability (P_d) is variable among tissues, and usually has a low value (about 10^{-5} m s^{-1}) (Tyreman et al. 1999). Transport of water

across pure lipid membranes occurs solely by diffusion with the ratio of the coefficient of osmotic water permeability to diffusional permeability approximating unity ($P_{os}/P_d \approx 1$) (Tyreman et al. 1999). This process cannot be inhibited by $HgCl_2$ or other chemical inhibitors (Chrispeels and Agre 1994). The diffusion of water across the lipid bilayer is strongly affected by the organization of membrane lipid molecules and is characterized by high Arrhenius activation energy (E_a = 46 to 63 kJ mol^{-1}), indicating that greater water movement will occur through the lipid bilayer at higher temperatures when the lipid mobilities are increased (Fettiplace and Haydon 1980, Agre et al. 1993, Tyreman et al. 1999).

1.4.4.2. Channel-mediated water transport

In non-stressed plants, water-channels may represent the predominant pathway for water flow across the plasma membrane (Henzler and Steudle 1995, Steudle and Henzler 1995, Carvajal et al. 1996, Carvajal et al. 1998).

Channel-mediated water transport across plasma membrane is characterized by: high value of osmotic water permeability (P_{os} = 10^{-4} to 10^{-3} m s^{-1}), relatively low activation energy (E_a = 17 to 25 kJ mol^{-1}), and larger osmotic water permeability than diffusional water permeability ($P_{os}/P_d > 1$) (Tyreman et al. 1999).

Unlike diffusional water permeability, it is possible to inhibit osmotically driven water permeability by chemical reagents known to covalently modify cystein residues (Kjelbom et al. 1999). Mercurial and sulphydryl reagents block many aquaporins, but there are also exceptions (Tyreman et al. 1999, Otto and Kaldenhoff 2000). Blocking can

be usually reversed with scavenging agents such as 2-mercaptoethanol or dithiotreithol (DTT) (Carvajal et al. 1999, Tyreman et al. 1999). The complete reversible effect of mercuric chloride on the *Chara* cell membrane suggested that about 75% of the water transport across the plasma membrane is mediated by specific water channels (Henzler and Steudle 1995).

1.5. Aquaporins

1.5.1. General

Every living cell must deal with osmotic and hydrostatic pressure changes in its environment (Heymann and Engel 1999). Our understanding of the mechanisms that cells employ to transport water and maintain turgor has been drastically improved by the discovery of membrane proteins that serve as channels for water and other small nonionic molecules (Heymann and Engel 1999).

The discovery of aquaporins provided solid molecular basis for fast and reversible regulation of transmembrane water transport and gave strong support to the idea that such high water permeability might be required for certain physiological processes (Maurel and Chrispeels 2001).

Aquaporins, or water channel proteins, belong to a highly conserved group of membrane proteins called the major intrinsic proteins (MIPs), with molecular weights in the range of 26-34 kDa (Tyreman et al. 2002). These proteins have been identified from numerous organisms ranging from bacteria, fungi to higher plants and animals (Ishibashi

and Sasaki 1998, Heymann and Engel 1999, Zardoya and Villalba 2001). The family includes more than 150 membrane channel proteins (Heymann and Engel 1999).

1.5.2. Structure

The structure of aquaporins is highly conserved among various types of organisms. The protein consists of 6 transmembrane helices with the N- and C-termini facing the cytosol. There are five loops (A-E) joining the transmembrane helices. The first cytosolic loop and the third extracytosolic loop are hydrophobic and contain the conserved asparagine-proline-alanine (NPA) sequence (Kjelbom et al. 1999, Tyerman et al. 2002). The two halves of the protein show obverse symmetry, with the hydrophobic loops containing the NPA motif overlapping in the middle of the lipid bilayer to form two hemipores that together create a narrow channel proposed to be similar in shape to an hour-glass (Jung et al. 1994, Tyerman et al. 2002).

Aquaporin polypeptides generally form homotetramers in the membrane (Daniels et al. 1999, Engle et al. 2000, Fotidias et al. 2001), with each monomer forming a single water pore (Jung et al. 1994). Water is thought to flow across the water channel pore in either direction down its potential gradient (Maurel 1997).

1.5.3. Plant aquaporins

The physiological role of water channel proteins is particularly important in plants because of their continuous water recruitment (Chrispeels and Maurel 1994, Kjelbom et

al. 1999).

There are two highly important aspects of plant aquaporins (Maurel and Chrispeels 2001). One aspect is their amazing diversity in plants: in *Arabidopsis* more than 30 genes encode aquaporin homologues (Weig et al. 1997, Maurel and Chrispeels 2001). The second aspect is the discovery that some aquaporins are multifunctional proteins, allowing the permeation of glycerol, CO_2, ammonia (NH_3), urea, boron, and hydrogen peroxide (Gerbeau et al. 1999, Henzler and Steudle 2000, Niemitz and Tyerman 2000, Ciavatta et al. 2001, Dordas and Brown 2001, Tchernov et al. 2001).

While all known aquaporins of mammalian cells have been localized to the plasma membrane, plant cells contain aquaporins in both the plasma membrane and tonoplast Kjellbom et al. (1999). However, Ma et al. (1993) reported that the mammalian aquaporin CHIP28 was also expressed in Golgi and endoplasmic reticulum (ER). Regarding their plant counterparts, NOD26 is located in the peribacteroid membrane of soybean nodule cells (Weaver et al. 1991), and, as suggested in a study by Kikuyama and Tazawa (1998), aquaporins could be located in the ER membrane of *Nitella flexis* plants.

The plant MIPs cluster in one of the following four classes: the tonoplast intrinsic proteins (TIPs) (Daniels et al. 1996, Karlsson et al. 2000), the plasma membrane intrinsic proteins (PIPs) (Johansson et al. 1996, Suga et al. 2001), the nodulin like plasma membrane intrinsic proteins (Kjellbom et al. 1999), and the small intrinsic proteins (SIP) (Siefritz et al. 2002). Most of the plant TIPs have been sequenced and so far are grouped into three subfamilies: αTIP, γTIP, and δTIP (Kjellbom et al. 1999, Zardoya and Villalba 2001). The plasma membrane aquaporins (PIPs) separate out into two subfamilies: PIP1 with low water channel activity and PIP2 with high water channel activity (Chaumont et

al. 2000).

The presence of water channels confers on an organism the possibility of accelerating the movement of water across membranes while diffusion still works in parallel (Schäffner 1998, Tyreman et al. 1999). Their activity may be required for fine regulation, which is influenced by cell metabolism, for example, via phosphorylation of the aquaporin proteins (Maurel et al. 1995, Tyreman et al. 1999).

Once a protein is involved, the cell has the ability to regulate its abundance (transcriptional or post-transcriptional) or to modulate its activity (Maurel and Chrispeels 2001). Water permeability of the plasma membrane and tonoplast through the aquaporins depends on their quantity, activity, and substrate specificity (Suga et al. 2001).

1.5.4. Aquaporin abundance

1.5.4.1. Diurnal and circadian fluctuations

In *Lotus japonicus* roots, the PIP1-type water channel showed diurnal fluctuations that were correlated with the diurnal variation in root hydraulic conductivity (Henzler et al. 1999). Harmer et al. (2000) found that the mRNA level of an *Arabidopsis* tonoplast aquaporin, δTIP, cycled in a circadian manner. The plasma membrane aquaporin in the motor cells of *Samanea saman* also showed a diurnal and a circadian regulation (Moshelion et al. 2002).

1.5.4.2. Aquaporin expression

Differential expression of the various aquaporin isoforms may regulate water flux across the plasma membrane (Johansson et al. 1998, Fotiadis et al. 2001). In spinach, the expression of So-δTIP was induced at the time when the large vacuole was formed (Karlsson et al. 2000). In *Arabidopsis thaliana*, γ-TIP mRNA accumulated at the time of vacuolation and subsided as the cells reached their full size (Ludevid et al. 1992).

The expression of aquaporin genes may be independently regulated in an organ- and stage-specific manner, while the amount of aquaporin protein can be regulated at the translational level and by the rate of protein turnover (Suga et al. 2001).

Studies regarding the aquaporin mRNA abundance have led to the following conclusions: 1) many aquaporins are highly expressed in the vascular tissues; 2) some tonoplast aquaporins are highly expressed in meristems, where vacuolar biogenesis takes place; 3) aquaporins are highly expressed in the tissues that can experience high water or metabolite flux (Barrieu et al. 1998, Chrispeels et al. 1999).

1.5.4.3. Cellular localization

In animal kidney, vasopressin induces large increase in water permeability of the apical membrane of principal cells in the collecting duct by the incorporation of vesicles containing AQP2 aquaporin (Nielsen and Agre 1995). Based on the distribution of PIP proteins within plant cells, Kirch et al. (2000) suggested that the control of plant water channel activity might appear through an analogous vesicle shuttle mechanism. It is

possible that the subcellular localization (in the plasma membrane or intracellular vesicles) of aquaporin tetramers is dependent on the level of phosphorylation of the subunits (Morillon and Lassalles 2002).

Höfte et al. (1991) found that a presumptive tonoplast aquaporin (*Phaseollus* α-TIP) is located in multivesicular bodies in transgenic tobacco that expresses *Phaseollus* α-TIP. Aquporins have been also localized in plasmalemmasomes, invaginating domains of the plasma membrane that protrude into the vacuole (Robinson et al. 1996, Fragne et al. 2001). As suggested by Robinson et al. (1996), clustering of aquaporins in the plasmalemmasomes may provide the means for achieving a rapid osmotic balance, and therefore, turgor maintenance in mesophyll cells.

The fact that the transport of solutes through the plasmodesmata can also occur through the desmotubules (Cantrill et al. 1999), raised the possibility that water may also take the desmotubule pathway via the endoplasmatic reticulum lumen (Tyreman et al. 2002).

1.5.5. Aquaporin activity

1.5.5.1. Phosphorylation

A number of studies indicate that the activity of aquaporins may be regulated by phosphorylation. When phosphorylated, some aquaporins display higher water channel activity (Maurel et al. 1995, Johansson et al. 1998, Harvengt et al. 2000).

As demonstrated by Johansson et al. (1996, 1998), species- and stress-specific

changes in cell hydraulic conductivity may be caused by phosphorylation of aquaporins. Phosphorylation of the plasma membrane aquaporin PM28A is carried out by a Ca^{2+}-dependent membrane-bound protein kinase and depends also on the apoplastic water potential (Johansson et al. 1996, Johansson et al. 1998).

Both tonoplast and plasma membrane aquaporins have putative phosphorylation sites that are conserved among different members of this family (Kjelbom et al. 1999).

1.5.5.2. Effect of turgor and osmotic pressure

There is no direct evidence for mechanosensitivity of aquaporin proteins, although some studies illustrate the potential for MIPs to be involved in volume or turgor homeostasis in plants (Steudle et al. 1982, Hohman et al. 2000, Tyreman et al. 2002). The dependence of mercury-induced closure of water channels on tissue turgor (Hejnowicz and Sievers 1996) indicates that changes in turgor can induce changes in the conformation of the plasma membrane, or even in the aquaporins themselves.

The decline of water permeability in *Chara* with increasing osmotic pressure has been suggested to be a consequence of pore dehydration in the membrane (Steudle and Tyerman 1983). Niemitz and Tyerman (1997) showed that the osmotic permeability of tonoplast-enriched vesicles declined with increased external osmotic pressure.

1.5.5.3. Effect of pH

There is not much known regarding the pH regulation of aquaporins. However, several studies suggested the involvement of pH in regulating the aquaporin activity. The reversible inhibition of water transport by acidic pH found in *Arabidopsis* plasma membrane (PM) vesicles by Gerbeau et al. (2002), was attributed to the blocking of aquaporins by H^+. In contrast, acidic pH activated the water conductance of the mammalian aquaporin AQP0 (Nemeth-Cahalan and Hall 2000). The water permeability of outer cortex cells of an acid sensitive maize variety was decreased by acidic pH, while no effect was recorded in a variety that was acid tolerant (Gunsé et al. 1997). On the other hand, there is no information so far on the effect of alkaline medium on the activity of aquaporins.

1.5.5.4. Effect of cations

Mercury (Hg^{2+}) has been used extensively to provide evidence for the involvement of aquaporins in water transport process in animal and plant cells. Mercury is thought to bind to sulphydryl groups of the aquaporin proteins, physically blocking the channels and reducing their hydraulic conductivity (Tyreman et al. 1999). Partial recovery of the water flow rate following the application of mercuric chloride was also observed in tomato and aspen root systems, implying the presence of aquaporins as the regulators of plant water status (Maggio and Joly 1995, Wan and Zwiazek 1999). However, the inhibition of water flow with mercurial reagents is neither completely understood nor a general characteristic

of aquaporins (Schäffner 1998). Some mercurial reagents, especially mercuric chloride, are highly membrane-permeant and are powerful metabolic inhibitors. That is why the effect of $HgCl_2$ on water permeation across the living cells should be interpreted with caution (Tyreman et al. 1999), since a possible outcome of $HgCl_2$ application could be the reduced phosphorylation of water-channels. As proven by Barone et al. (1997), mercury can also induce conformational changes in the plasma membrane aquaporins of higher plants.

Calcium ions (Ca^{2+}) play a central role in cell signal transduction and are involved in responses to stresses such as hypoxia and salinity (Sanders et al. 1999). Aquaporins in plant membranes can undergo Ca^{2+}-dependent phosphorylation, which can increase their water-channel activity (Maurel et al. 1995, Johansson et al. 1998, Johansson et al. 2000). On the other hand, Ca^{2+} decreased the osmotic water permeability of PM vesicles from *Arabidopsis* (Gerbeau et al. 2002), suggesting a potential relevance to intracellular Ca^{2+} signaling. At the whole plant level, Ca^{2+} has been shown to ameliorate the reduction of root hydraulic conductivity produced by salinity (Martinez-Ballesta et al. 2000).

Zinc (Zn^{2+}) inhibited the water transport across the membranes of *Chara* cells (Tazawa et al. 1996) and the water transport activity of *Actinidia deliciosa* protoplasts (Qiu et al. 2000). There is increasing evidence that zinc may be an integral part of the biomembranes, required for the stability and control of lateral mobility of membrane molecules (Rygol et al. 1992). As suggested by Tazawa et al. (1996), zinc might bind to the proteinaceous water-pore (aquaporins).

The effect of other cations on water channel activity has been tested. Sodium (Na^{+}), magnesium (Mg^{2+}), manganese (Mn^{2+}), potassium (K^{+}) had no effect on the water

transport activity of *Actinidia deliciosa* protoplasts (Qiu et al. 2000). On the other hand, Ba^{2+}, Mg^{2+}, and Sr^{2+} reduced the osmotic water permeability of *Arabidopsis* PM, suggesting a direct blockage of aquaporins by direct cation binding (Gerbeau et al. 2002). The Mg^{2+}-dependant protein kinase phosphorylates the lentil seed aquaporins (Harvengt et al. 2000). Magnesium (Mg^{2+}) can physically interact with the aquaporin proteins or with their neighbouring lipids as suggested by Ren et al. (2000), and Fu et al. (2000). In the case of Na^{+}, the decrease in membrane hydraulic conductivity has been attributed to sodium competition with calcium ions for membrane binding sites (Martinez-Ballesta et. al. 2000).

The effect of cations on osmotic water permeability of the plasma membrane is complex and not well understood. In plants exposed to environmental stimuli, there is a complex interaction of different cations with direct or indirect effects on the aquaporin water transport activity.

1.5.6. Plant hormones and aquaporins

It has been known for several years that abscisic acid (ABA) can have an effect on the aqueous permeability of the root cell membranes. Direct evidence from experiments with $HgCl_2$ (Quintero et al. 1999), a study by Hose et al. (2000) that used cell and root pressure probes, and the experiments by Morillon and Chrispeels (2001) that quantified the aquaporins from the cells with increased osmotic permeability, suggests that the effect of ABA is at the plasma membrane level, presumably by activating aquaporins. However, exogenous ABA applied to the roots of aspen seedlings closed the stomata but had no

effect on root hydraulic conductivity and on the activation energy of root water flow (Wan and Zwiazek 2001).

In elongating tissues, gibberellic acid has been shown to control the expression of a number of genes, including the tonoplast intrinsic protein, a water channel, which may regulate water fluxes into the vacuole during cell expansion (Huttley and Phillips 1995).

Ethylene can increase plasma membrane permeability permitting more water to cross the cells (Woltering 1990). At the whole plant level, increased water transport in hypoxic aspen seedlings exposed to ethylene was explained in terms of enhanced aquaporin activity, probably due to a direct effect of ethylene on the phosphorylation of aquaporins (Kamaluddin and Zwiazek 2002).

Brassinolide increased the osmotic permeability of hypocotyl protoplasts from *Arabidopsis thaliana* (Morillon et al. 2001), while natriuretic peptides, a new proposed class of plant hormones, promoted radial water movement out of the shoots of *Tradescantia multiflora* (Suwastika and Gehring 1998), both interpreted as the effect of aquaporin activation.

1.5.7. Water stress and aquaporins

Water stress can change the expression patterns of aquaporin genes (Tyreman et. al. 2002). Some of their genes are up-regulated in response to desiccation (Yamada et al. 1997, Barrieu et al. 1999), while others are down-regulated (Yamada et al. 1997, Smart et al. 2001).

Drought stress induced in the rice seedlings appeared to increase the physiological

functioning of water-channels by increasing the root water channel activity or by increasing the aquaporin number compared with unstressed control seedlings (Lu and Neumann 1999). Under drought conditions, the aquaporin PM28A in spinach leaves is dephosphorylated and therefore rendered inactive (Johansson et al. 1998). Since drought stress decreased the osmotic permeability of root protoplasts but did not have any influence on the amount of PIP1 and PIP2 aquaporins, it is plausible that drought affected the protein functionality (Morillon and Lassalles 2002).

Under water stress, the synthesis of ABA is stimulated which, in turn, can stimulate the activity of water channel proteins (Hose et al. 2000, Netting 2000, Wilkinson and Davies 2002).

In wheat roots, hypoxia caused similar effects as $HgCl_2$ on water uptake and hypoxia-treated cells could not be further inhibited by $HgCl_2$ suggesting an effect on the water channel activity (Zhang and Tyreman 1999).

1.5.8. Effect of nutrient stress

The reduction of root hydraulic conductivity and its loss of mercury sensitivity in wheat plants affected by N-, P- and S deficiencies, suggested that either the activity or the density of water channels in the root cell plasma membrane is diminished during nutrient deficiency (Clarkson et al. 2000).

Wang et al. (2001) found that nitrate can up-regulate expression of seven different water channel genes. However, the timing of up-regulation (48 hours), suggested that nitrate uptake triggered an increase in the symplastic solute concentrations, which, in

turn, stimulated water uptake (Wang et al. 2001).

1.5.9. Salt stress

Salinity can negatively affect root water uptake. The weak response of root hydraulic conductivity in salt-stressed roots of paprika pepper (*Capsicum annuum* L.) to $HgCl_2$ was interpreted in terms of reduced number or reduced functionality of water channel proteins (Carvajal et al. 1999). On the other hand, osmotic stress increased the abundance of mRNA of the tonoplast aquaporin in cauliflower (*Brassica oleracea* L. var. *botrytis*) cells (Barrieu et al. 1999). In a salt-tolerant variety of rice (*Oryza sativa* var. *Pokkali*), salinity stress up-regulated the transcripts encoding a putative water channel protein, while the transcripts encoding other water channel proteins were not changed during the stress period (Kawasaki et al. 2001).

1.5.10. Significance of aquaporins at the whole plant level

The high degree of conservation of water channel proteins from bacteria through plants and animals suggests their fundamental importance (King et al. 2000). In mammals, mutations that affect the aquaporin structure were implied to cause a variety of clinical conditions (King et al. 2000).

Aquaporins can provide spatial markers to explore the intricate flows of water and solutes that play a critical role throughout all stages of plant development or in responses of plants to biotic or abiotic stresses (Maurel and Chrispeels 2001). Two functions have

been postulated for the main two families of aquaporins present in plants based on their expression patterns: a role for TIPs in the intracellular osmotic equilibration, and a role for PIPs in the regulation of transcellular water transport (Kjelbom et al. 1999, Marin-Olivier et al. 2000).

There is growing evidence that suggests that aquaporins play different roles throughout plant development. During seed development in Norway spruce (*Picea abies*), high transcript expression of aquaporins was found in the spongy tissue encasing the developing female gametophyte, in the cells of the future seed coat of young ovules, and in the storage parenchyma cells of the gametophyte (Hakman and Oliviusson 2002). During seed germination, the fine tuning of TIP aquaporin activity may allow for controlling changes in the vacuolar volume (Maurel et al. 1997). Self-incompatibility response in *Brassica* that refers to the rejection of self-related pollen requires the expression of an aquaporin gene (Ikeda et al. 1997, Marin-Olivier et al. 2000). Crassulacean acid metabolism (CAM) plants control the expression of aquaporins at a low level to regulate the water flow in succulent organs, which may be advantageous for CAM plants in arid environments (Ohshima et al. 2001).

A powerful tool in elucidating the aquaporin function is given by the reverse genetics that can also reveal unexpected function of water channel proteins. Anti-sense plants of *Arabidopsis thaliana*, transformed with a construct targeting PIP1b aquaporin, showed an increase in root mass compared with the control plants (Kaldenhoff et al. 1998). Plants lacking PIP1b aquaporins and, therefore, having increased resistance to water movement in root cells, compensated for this effect by increasing their root surface area (Kaldenhoff et al. 1998). Anti-sense tobacco plants showed no morphological

changes, but instead, physiological changes were observed (Siefritz et al. 2002). In this study, the overall root hydraulic conductivity was reduced and, when plants were stressed with low soil water potential, anti-sense plants appeared to be less tolerant and were not able to maintain turgor. So far, it appears that aquaporins are important not only in determining the anatomical appearance of a plant but also in modulating the plant response to environmental stresses such as drought. The above data suggest that aquaporins play an important role in determining the hydraulic conductivity of plant tissues. Since roots can finely adjust their hydraulic conductivity in response to environmental changes, aquaporins could perfectly provide for such regulation (Tyreman et al. 1999). Consequently, the search for aquaporin function has given rise to new experimental approaches, mostly in the study of water uptake by the roots (Maurel and Chrispeels 2001).

1.6. Characterization of root water uptake

The ability of a root system to extract water from soil and to transport it to the shoots is primarily determined and characterised by root hydraulic conductivity, L_p (Huang and Nobel 1994).

Since the greatest resistance to liquid water flow in the soil-plant-atmosphere continuum (SPAC) is imposed by roots, the modelling of water flows across the root has received great interest (Zhu and Steudle 1991).

Water uptake by the roots is a variable process that depends on the species (Rieger and Litvin 1999), root structure and anatomy (North and Nobel 1995), time of the day

(Else et al. 1995, Henzler et al. 1999) and root age (Barrowclough et al. 2000). The process of water absorption by the root is also influenced by environmental factors such as drought (Lu and Newman 1999), flood (Lopez and Kursar 1999), temperature (Markhart 1984, Nobel et al. 1990), salts (Carvajal et al. 1999), and the availability of mineral nutrients (Clarkson et al. 2000). Consequently, root hydraulic conductivity is also affected by the above factors. The variation in root hydraulic conductivity may be a physical adjustment (e.g. the stress-induced changes in the anatomy of roots), or an active regulation of root hydraulic properties (Steudle 2000b).

1.6.1. Root hydraulic conductivity

Water flow through a root can be quantified as the root hydraulic conductance or root hydraulic conductivity.

The absolute hydraulic conductance (K, kg s^{-1} MPa^{-1} or m^3 s^{-1} MPa^{-1}) is the ratio of water flow rate (F, kg s^{-1} or m^3 s^{-1}) through the plant or plant segment to the pressure difference that cause this water flow (ΔP, MPa) (Becker et al. 1999):

$$K = \frac{F}{\Delta P} \qquad (\text{kg s}^{-1}\ \text{MPa}^{-1}) \text{ or } (\text{m}^3\ \text{s}^{-1}\ \text{MPa}^{-1})$$

The absolute hydraulic conductivity is the ratio of water flow rate (F, kg s^{-1} or m^3 s^{-1}) and the pressure gradient (dP) over a segment of length dx (dP/dx, MPa m^{-1}) causing the flow (Tyree and Ewers 1991):

$$k_h = \frac{F}{dP/dx} \quad (\text{kg s}^{-1}\ \text{MPa}^{-1}\ \text{m}) \text{ or } (\text{m}^3\ \text{s}^{-1}\ \text{MPa}^{-1}\ \text{m})$$

The absolute hydraulic conductance and conductivity do not express how well the root system supplies the shoot with water, and cannot be used to compare different root systems. Hydraulic conductance expresses the capacity of the whole root system to supply water to the shoot, and bigger roots usually have bigger conductances, for the same species and in the same conditions. Hydraulic conductivity, on the other hand, refers to the performance of a unit length of a root in providing water, but does not account for differences in the diameter between roots.

The root hydraulic parameters have been usually normalised by the root surface area (Wan et al. 2001), root length, root fresh weight (Carvajal et al. 1999), and root volume (Kamaluddin and Zwiazek 2002). The normalisation permits for a comparison between different root systems.

Determination of the parameters that characterize the water movement across roots usually involves measuring the volume of water flowing through a root that is exposed to a known water potential gradient (Huang and Nobel 1994). The forces that induce water flow through the root can be hydrostatic or osmotic. When an externally applied positive hydrostatic pressure (ΔP) is increased in small steps, the water flux from detopped roots increases curvilinearly at first and then linearly at higher pressures (Jackson et al. 1996). Root hydraulic conductance is determined from the slope of the linear part of the plot of water flow rate versus applied pressure (Tyree et al. 1995). There are some discrepancies among osmotically determined root hydraulic parameters and the hydrostatically

determined ones, possibly due to different pathways for radial water movement, interaction between water and solute flow or the existing osmotic pressure gradient (Zhu and Steudle 1991).

In intact plants, the sap flow is a consequence of driving forces comprising the balance between opposing osmotic forces ($\Delta\pi$) created by solutes dissolved in soil water and xylem sap, and a negative hydrostatic pressure (ΔP) generated by evapo-transpiration (Markhart and Smit 1990). Therefore, root hydraulic conductance K ($m^3\ s^{-1}\ MPa^{-1}$) is determined from the following relation (Fiscus 1975, Passioura 1988):

$$Q_v = K\ (\Delta P - \sigma\Delta\pi)$$

where Q_v ($m^3\ s^{-1}$) is the flow rate of water through the root, and σ is the reflection coefficient for solutes.

For intact plants, root hydraulic parameters can be estimated using the rate of transpiration or water uptake (Saliendra and Meinzer 1992).

Water flow though the root is usually described by the root hydraulic conductivity (L_p) (Huang and Nobel 1994, Wan and Zwiazek 1999) defined as:

$$L_p = (1/A)\ (Q_v/\Delta P)\ \ (m^3\ s^{-1}\ MPa^{-1}\ m^{-2})$$

where A (m^2) is the root surface area, Q_v ($m^3\ s^{-1}$) is the volumetric flow rate of water, and ΔP (MPa) is the pressure drop along the root. Looking at the above equation, hydraulic conductivity may also be viewed as the absolute hydraulic conductance expressed on the root surface area basis. Some studies refer to L_p calculated based on the above equation as the hydraulic conductance (Lo Gullo et al. 1998). However, hydraulic conductance (K) is usually considered to be converted to hydraulic conductivity (L_p) by dividing K by the root surface area (Markhart and Smit 1990).

1.6.2. Components of root hydraulic conductivity

According to Rieger and Litvin (1999), water uptake by the whole root system is a function of two parameters: the root hydraulic conductivity and the offset, calculated as the intercept of the plot of water flow rate versus water potential gradient, to the water potential gradient axis. The offset is independent of the root hydraulic conductivity (Rieger and Litvin 1999). The offset has been interpreted as the minimum water potential gradient across the roots required to initiate flow (Steudle 1994) and it appears to be a real component of root hydraulics related to apoplastic barriers in the root such as the presence of an exodermis (Rieger and Litvin 1999).

Water moves within a root along a radial pathway followed by a longitudinal pathway. As a consequence, root hydraulic conductivity (L_p) can be separated in two components: the radial conductivity for the flow from the root surface to the xylem, and the axial conductivity for the flow along the xylem (Nobel et al. 1990, Huang and Nobel 1994, Doussan et al. 1998a). According to the composite model of water movement into roots, the radial conductivity of the root consists of an apoplastic component and a cell-to-cell component, while the axial conductivity accounts mainly for apoplastic flow through the xylem. Because the longitudinal pathway is constituted by dead elements, the hydraulic properties of the conduits determine the transport of water at this level. The radial pathway is very important, because in order to get into the conducting elements, water has to pass through or between more layers of cells, making the water movement more likely prone to metabolic influences.

The relative importance of the two components of root hydraulic conductivity varies with plant species and the developmental stage of the root (Huang and Nobel 1994). The modelling of the root system water uptake for maize (*Zea mays*) shows that during root tissue maturation, the radial conductivity decreases by one order of magnitude while the axial conductivity increases by about three orders of magnitude (Doussan et al. 1998b).

The main resistance to water flow in a small root system is thought to reside in the non-vascular pathway where water moves radially from the root surface to the vessels in the stele (Frensch and Steudle 1989). According to Huang (2000), differences in the root hydraulic conductivity among citrus rootstock (*Citrus* sp.) were mainly related to structural differences in the radial pathway for water movement, suggesting that the radial hydraulic conductivity was the primary determining factor for water uptake. It is estimated that, in the radial pathway, 70-90% of the resistance to water flow is imposed by the living tissue located between the ambient solution and the xylem vessels, whereas the remaining resistance is due to water transfer across the vessel walls (pits) (Steudle and Peterson 1998).

Along the developing onion roots, resistances to water movement are variable and are concentrated in certain layer or layers of the radial pathway (Barrowclough et al. 2000). Due to the anatomy of roots, the main resistance for water flow in the apoplastic pathway is determined by the development of the suberin lamellae and Casparian bands (Steudle 2000a). Root hydraulic conductivity is inversely related to root cortical thickness and to the presence of a suberized exodermis (Rieger and Litvin 1999). The limitation imposed on root hydraulic conductivity by the suberized layers increases during root

development and even more during soil drying (North and Nobel 1995).

The contribution of different pathways to the overall radial water flow can be estimated by a double pressure probe technique (Zhu and Steudle 1991). Water flow through the cell-to-cell pathway and its hydraulic conductivity can be measured in individual cells using a cell pressure probe (Steudle 1993).

Root hydraulic conductivity, the axial and radial components are generally genetically determined, but can be modified by environmental factors, such as soil water availability and temperature (Huang and Nobel 1994). There is also a diurnal variation of the root hydraulic conductivity: it declines toward the end of the light period than rises again at the end of the dark period and has a maximum during the day (Parson and Kramer 1974, Else et al. 1995, Henzler et al. 1999). Root hydraulic conductivity also decreased with increasing root age for the roots of *Agave deserti* (Nobel et al. 1990).

1.6.3. Environmental stress effects on root hydraulic conductivity (L_p)

1.6.3.1. Water stress and root hydraulic conductivity

Water stress is determined by an insufficient or excessive water activity in the plant's environment (Levitt 1980). There are two types of water stresses: 1) drought stress, also referred to as water stress or water deficit stress, and 2) excess water stress or flooding stress. Both stresses usually have a negative impact on plant water relations. Root hydraulic conductivity is usually diminished in both flooded- or drought-stressed plants (Smit and Stachowiak 1988, Bradford and Hsiao 1982, Lo Gullo et al. 1998,

Steudle 2000a).

Drought stress. Drought stress occurs when available water in the soil is reduced and atmospheric conditions cause continued loss of water by evapo-transpiration (Hale and Orcutt 1987).

Root hydraulic conductivity decreases in plants subjected to drought stress (Nobel and Lee 1991; Lo Gullo et al. 1998; Lu and Newman, 1999, Rieger and Litvin 1999). During soil drying, changes in root hydraulic conductivity represent a combination of changes in the conductivity of individual root tissues, including a reduction in the relative contribution of the apoplastic pathway to radial water flow (North and Nobel 1996).

The reduction of the hydraulic conductivity of the root systems subjected to drought has been attributed to xylem cavitation in cotton (Byrne et al. 1977), decreased cell permeability in lemon and citrus (Levy and Syvertsen 1983) or increased resistance at the soil-root interface (Faiz and Weatherly 1978). Studies with sugarcane (*Saccharum* sp.) suggested that changes in hydraulic properties within the root system occurred in response to soil drying (Saliendra and Meinzer 1992). In dry soils, roots can also shrink radially and form air gaps between the root and the soil, which by interruption of the liquid-phase continuity can limit the water movement (Taylor and Willatt 1983). The radial shrinkage of roots also affects the radial pathways of water movement and the longitudinal path which, in turn, may result in a greater resistance for water movement and lower hydraulic conductivity in drought-stressed roots. The importance of the endodermis, in addition to being a barrier for water movement, resides in controlling root shrinkage during soil drying (Saliendra and Meinzer 1992).

Usually, drought reduces root hydraulic conductivity by inducing changes in root

anatomy through suberization (North and Nobel 1995). Following drought, air lacunae also form in the cortex, which may contribute to the reduced radial conductivity (Rieger and Litvin 1999).

Seasonal changes of root hydraulic conductance have been shown to account for the different drought resistance strategies adopted by forest trees under water stress conditions (Nardini and Pitt 1999). Some species that avoid drought by reducing water loss, apparently increase root hydraulic conductance during dry and hot summers (Nardini et al. 1998). Drought-tolerant species that can function under conditions of low tissue hydration appear to reduce root and stem hydraulic conductance (Lo Gullo et al. 1998). The ability to maintain efficient vertical water transport under water stress conditions seems to be a key factor in distinguishing drought-avoiding from drought-tolerant species (Nardini and Pitt 1999).

Drought stress is also known to increase the concentration of ABA in roots (Zhang et al. 1987). The ABA increases the root hydraulic conductivity of aeroponically grown maize to values of roots lacking an exodermal layer (Freundl et al. 2000), possibly by affecting the function of water-channels (Tyreman et al. 1999).

Flooding stress. Flooding is defined as the presence of soil water in excess of the soil field capacity. Flooding stress affects plants by inducing secondary stresses including: oxygen deficiency stress, CO_2 and ethylene stress, ion stress, and water-deficit stress (Levitt 1980). Therefore, the effect of flooding on root hydraulic conductivity may be determined by the secondary flood-induced stresses and by their interactions.

Water excess or hypoxic stress reduced the root water uptake in tomato plants (Else et al. 1995), hybrid polar (Smit and Stachowiak 1988), and oak and beach seedlings

(Schmull and Thomas 2000). However, ten days of flooding caused little change in the root hydraulic conductivity of eastern larch and white spruce (Reece and Riha 1991). Root hydraulic conductivity can also increase following flooding in tomato plants (Bradford and Hsiao 1982, Else et al. 1995) and three different tropical tree species (Lopez and Kursar 1999).

The increased resistance to water flow in flooded roots may occur by an occlusion of the xylem vessels (Andersen et al. 1984). However, the depression of conductivity may be mediated by conformational changes in the water channel proteins (Else et al. 1995). The increase in root hydraulic conductivity indicates a sudden loss of the resistance mechanisms, probably an outcome of membrane degeneration (Else et al. 1995) or a possible leakage of water into the root due to increased root tip dieback (Lopez and Kursar 1999). Aerenchyma development may increase internal root aeration sufficiently to reverse the anaerobically-induced reduction in membrane permeability to water (Harrington 1987). Flooding increases the osmotic potential of the xylem sap, adding a supplementary osmotic driving force, which can explain the similar hydraulic conductivities measured in aerated and flooded roots (Jackson et al. 1996).

According to Gibbs et al. (1998), in roots exposed to oxygen deficiency conditions, the reduction in root hydraulic conductivity is not a principal effect of hypoxia, and long-term changes of water fluxes in O_2 deficient roots may be an indirect, rather than direct effect of oxygen deficiency on roots.

Roots exposed to flooding also experience morphological changes that could have an impact on the root hydraulic conductivity. Adventitious roots produced in response to flooding represent a supplementary absorbing system and compensate for the loss of root

function associated with the decay of some of the original roots (Sena-Gomes and Kozlowski 1980).

1.6.3.2. Salt stress and root hydraulic conductivity

Salt stress may reduce plant growth by inducing water deficit, ion toxicity, ion imbalance, or a combination of these factors (Cramer and Jones 1996). Plants grown under salt stress reduce their internal osmotic potential in order to achieve water uptake and maintain turgor, but high levels of salts, especially NaCl, have been reported to reduce hydraulic conductivity (Azaizeh and Steudle 1991, Carvajal et al. 1999, Carvajal et al. 2000).

1.6.3.3. Low temperature and root hydraulic conductivity

Low temperature decreases root hydraulic conductivity relatively rapidly (Bolger et al. 1992, Bigot and Boucaud 1994, Wan et al. 1999, Wan et al. 2001). The decrease of root hydraulic conductivity has been explained through an increase in water viscosity (Kimmins 1996) and by the effect of low temperature on cell metabolism and membranes (Zhang and Tyreman 1991).

In aspen seedlings, the inhibition of root water uptake by low temperatures could not be fully explained by increased water viscosity (Wan et al. 2001). It was suggested that water channel proteins are involved in the reduction of root water uptake induced by low temperature and the effect is probably mediated through root respiration (Wan et al.

2001, Kamaluddin and Zwiazek 2001).

1.7. Inhibitory studies of root water uptake

Short-term changes in root water uptake or in the rate of water flow have been obtained with different chemical inhibitors. In most cases, these studies have been used to infer the existence and the importance of aquaporins in regulating water fluxes across the roots, as well as aquaporin activity regulation or presumable links between the aquaporins and different cellular processes.

Mercury compounds have been used to study the metabolism and transport processes within a plant (Eckert et al. 1999). Reversible inhibition of water flow by mercurials has been obtained for membrane vesicles (Maurel 1997, Tyreman et al. 1999), root protoplasts (Zhang and Tyreman 1999), and tomato and aspen root systems (Maggio and Joly 1995, Wan and Zwiazek 1999).

Studies using respiration inhibitors or stresses that resulted in both decreased water uptake and respiration, pointed out that the functioning of aquaporins is linked to respiration, possibly through the supply of ATP for aquaporin phosphorylation (Zhang and Tyremann 1991, Kamaluddin and Zwiazek 2001, Wan et al. 2001).

Inhibition of water transport across onion roots by K^+ channel inhibitor, suggests that the fluxes of K^+, one of the essential factors, are linked to the activity of water channels in the plasma membrane (Tazawa et al. 2001).

1.8. Composite model of water uptake versus protein dependent water uptake

Plant roots have a remarkable capacity to alter their L_p over the short term in response to many stimuli, such as day/night cycles, nutrient deficiency or stress, and before any changes in root anatomy and morphology can be observed (Javot and Maurel 2002).

The variation of root hydraulic conductivity has been interpreted with respect to changes in water flow through several pathways across the root (Steudle and Peterson 1998, Tyreman et al. 1999). In a composite transport model of the root, in the presence of hydrostatic gradients, water flow occurs mostly through apoplast, whereas in the absence of hydrostatic forces water will flow through the cell-to-cell pathway (Steudle 1994). Changes in root resistance could be caused by a combined effect on the apoplastic and cellular paths (coarse and fine regulation) (Zimmermann et al. 2000), which may provide a useful mechanism by which a plant can respond to changing environmental conditions (Tyreman et al. 1999). In the view of the composite model of water transport, the plant can switch between the different pathways by allowing an adjustment of root hydraulics according to the demands from the shoot (Steudle and Peterson 1998). The presence of water channels confers on the organism a possibility of accelerating the movement of water across membranes while diffusion still works in parallel as required for the fine regulation of water uptake (Maurel et al. 1995, Schäffner 1998, Tyreman et al. 1999). However, the predominance of one path over the other may also depend on a plant species.

According to the composite model, under normal conditions and in the absence of

anatomical changes, water moves in transpiring plants mostly through the apoplast. Yet, the studies with maize roots under hypoxic conditions argued strongly against the hypothesis that water flows predominantly through the apoplastic pathway in response to a hydrostatic driving force (Gibbs et al. 1998). Diurnal variations in root hydraulic conductivity are correlated with the diurnal fluctuation in the expression of aquaporins in the roots of *Lotus japonicus*, implying that water channels may contribute to the root hydraulic conductivity and its fluctuation within a day (Henzler et al. 1999). The accumulating evidence implies that in most plants, and in contrast to what was suggested in earlier studies, the cell-to-cell pathway plays a major role in the overall root water uptake (Javot and Maurel 2002). Inhibitor studies using whole root systems and the pressure-flux technique suggest that in higher plants, water flows across the roots predominantly through the cell-to-cell pathway in response to hydrostatic pressure (Wan and Zwiazek 1999, Kamaluddin and Zwiazek 2001).

It is still unknown where exactly aquaporins are concentrated or activated in the root. It was suggested that the regulation of root water flow that involves aquaporins, takes place mostly in the membranes of stele cells that surround the vessels (Henzler et al. 1999). However, new data shows that, in young maize roots, the epidermal cell layers have an essential role in the control of water transfer (Gaspar et al. 2001).

In plant roots, the predominant function of the aquaporins seems to be facilitating water uptake (Javot and Maurel 2002). The importance of aquaporins in the regulation of water flow across roots suggests that the composite model for water transport may need to be modified. The elements that allow for the perception of environmental stimuli by roots and subsequent aquaporin regulation are little known and still require more

investigation (Javot and Maurel 2002).

1.9. Calcium and calmodulin

Calcium plays a key role in plant growth and development. Changes in cellular Ca^{2+}, acting through Ca^{2+}-modulated proteins and their targets, regulate an astonishing variety of cellular processes (Bush 1995). Calcium is involved in auxin binding, auxin transport, auxin-induced elongation, cell wall material secretion, tropisms, cell division, osmoregulation, cation transport, guard cell swelling, freezing injury, cell polarity, photosynthesis and a number of other physiological processes (Poovaiah and Redd 1987, Sanders et al. 1999).

Cellular Ca^{2+} homeostasis is maintained by various Ca^{2+} transport proteins: those that mediate efflux from the cytoplasm (the Ca^{2+}-ATPase and Ca/nH^{+} antiporters) and those that mediate influx into the cytoplasm (the Ca^{2+} channels) (Bush 1995).

An important function of Ca^{2+} is its involvement in stimulus-response coupling in all eukaryotic organisms. Calcium is not the only regulator of cellular functions, but any stimulus that induces a complex cellular response will likely involve Ca^{2+} at some point in the transduction pathway (Bush 1995).

The best characterized calcium-modulated proteins from plants are calmodulin and the calcium dependent calmodulin-independent protein kinase (CDPK) (Roberts and Harmon 1992). Calmodulin is a highly conserved calcium-modulated protein that appears to be ubiquitous among eukaryotes (Roberts et al. 1986). It has no enzymatic activity of its own, but it modulates the activity of several enzymes and some non-enzymatic

proteins in a calcium-dependent manner (Roberts and Harmon 1992). Having a wide range of functions, calmodulin is thought to be a fundamental component of calcium signal transduction pathways. The classes of calmodulin-stimulated enzymes and calmodulin-binding proteins include NAD kinase, protein kinases, Ca^{2+}-ATPase and transport proteins (Roberts et al. 1986).

1.10. Protein phosphorylation in plants

Change in the activity of enzymes or other proteins by their phosphorylation and dephosphorylation is used to control many cellular activities (Hunter 1995). In particular, protein phosphorylation is the major component of signal transduction pathways (Hunter 1995).

Hunter (1987) pointed out that a protein kinase-phosphatase cycle was analogous to a transistor, and like the latter, it can act as a switch, changing the function of target protein on or off, and/or as an amplifier, increasing the amplitude of a signal. However the network of protein kinases and phosphatases within the cell can be likened to the assemblage of transistors in the central processor unit (CPU) of a computer, accepting information from receptors that sense environmental conditions, phytohormones, and other external factors, and converting this information into appropriate outputs such as changes in metabolism, membrane transport, gene expression, cell growth and division, development and differentiation (Hardie 1999, Hardie 2000). The analogy is imperfect because most of the kinase-phosphatase switches work in analogue fashion and are capable of intermediate states between off an on (Hardie 2000).

Basically phosphorylation and dephosphorylation of proteins involves the following reactions:

Phosphorylation

$$\text{Protein} + n\text{ATP} \xrightarrow{\text{protein kinases}} \text{Protein-Pn} + n\text{ADP}$$

Dephosphorylation

$$\text{Protein-Pn} + H_2O \xrightarrow{\text{protein phosphatases}} n\text{Pi} + \text{Protein}$$

Phosphorylation of eukaryotic proteins occurs predominantly (97%) on serine and threonine residues and to a lesser extent on tyrosine residues (Shenolikar 1994). In plants, protein phosphorylation and dephosphorylation play important roles not only in cell development but also in responses to stresses (Ichimura et al. 2000, Hardie 2000).

1.10.1. Plant protein kinases

Enzymes of the eukaryotic protein kinase superfamily catalyze the reversible transfer of the γ–phosphate usually from ATP to amino acid side chains of proteins (Stone and Walker 1995). Some of these enzymes play pivotal roles in the control of plant defence mechanisms, signal transduction, and metabolism (Smith and Walker 1996).

Typically, eukaryotic protein kinases have been subdivided into kinases that phosphorylate a) serine and/or threonine, b) tyrosine, and c) histidine residues.

The plant protein kinase family can be grouped into about 14 subfamilies. The major subfamilies are the receptor-like kinases (RLKs), the GSK3/shaggy subfamily, the MAP kinases (MAPKs), MAP kinase kinases (MAPKKs), MAP kinase kinase kinases (MAPKKKs), the calcium-dependent protein kinases (CDPKs) and Snf1-related kinases (SnRKs) (Hardie 2000). In plants, the protein kinases that are implicated in the early events of signal transduction are mediated by unique protein kinases, but these signals converge into pathways utilizing more conserved protein kinases that are universal in eukaryotes (Stone and Walker 1995). Protein kinases are responsible for signal recognition (RLK), but there are also second-messenger-regulated protein kinases (CDPKs), which have a calcium-binding, calmodulin-like domain (Stone and Walker 1995).

There is large variety of proteins that can be phosphorylated in vivo by CDPK including nitrate reductase, sucrose synthase, tonoplast intrinsic protein (an aquaporin), K^+ and Cl^- channels from guard cell tonoplasts, and plasma membrane H^+-ATPase (Hardie 2000, Hrabak 2000).

In animals, the mitogen-activated protein kinases form cascades operating in the direction MAPKKK→MAPKK→MAPK. In plants, MAPK kinase pathways are involved in the ethylene hormonal response, they mediate abiotic and biotic stress responses, and are involved in the cell cycle regulation (Jouannic et al. 2000).

The RLK family includes tyrosine protein kinase with receptor-like domains that have a cytoplasmatic catalytic domain, a membrane-spanning region, and an extracellular

domain (Torii and Clark 2000). In plants, these kinases are involved in regulation of organ shape, maintenance of the shoot apical meristem, brassinosteroid signalling, specification of the epidermis, pollen development, osmotic stress and light regulation (Torii and Clark 2000).

1.10.2. Plant protein phosphatases

Protein kinase function can be countered by the action of protein phosphatases. Protein phosphatase activities have been reported in most plant subcellular compartments, including mitochondria, chloroplasts, nuclei, and cytosol (Smith and Walker 1996).

Since some plant and animal protein phosphatases are highly conserved, their classification has been based on the animal classes of protein phosphatases. There are three big families of protein phosphatases: protein phosphatase P (PPP: PP1, PP2A, PP2B, and PP2C) and protein phosphatase M (PPM: Mg^{2+}-dependent protein phosphatases) families that are serine/threonine phosphatases, and the protein tyrosine phosphatases (Cohen 1997, Barford et al. 1998).

In yeast and mammalian systems, four major Ser/Thr phosphatase activities have been detected and classified according to their composition, substrate selectivity, inhibitor sensitivity, and absolute requirement for bivalent cations (Dong et al. 2001). PP1 dephosphorylates the β-subunit of the mammalian phosphorylase kinase preferentially and is inhibited by inhibitor-1 and inhibitor-2. PP2 has greater preferential activity toward the α-subunit of the mammalian phosphorylase kinase and is resistant to inhibitor-1 and inhibitor-2. It can be divided further into three subgroups: PP2A (does not

require divalent cations for activity), PP2B (a Ca^{2+}/calmodulin activated phosphatase), and PP2C (activity requires Mg^{2+}).

Homologues of the four major serine/threonine phosphatases groups have been detected in plants.

Protein phophatase-1 (PP1) is a ubiquitous and highly conserved enzyme found in all eukaryotes (Smith and Walker 1996). In *Brasica napus* seed extracts, the PP1 activity was associated predominantly with membranes of the endoplasmic reticulum and other unidentified particulate fractions (MacKintosh at al. 1991). PP1 is almost exclusively cytosolic in pea leaves and carrot cells and is associated with microsomes in wheat leaves (MacKintosh at al. 1991). It has also been found in isolated nuclei and plasma membranes (Vera-Estrealla et al. 1994, Sheen 1993).

Protein phophatase-2A (PP2A) is found in most tissues and in many subcellular locations including nucleus and cytoplasm and is associated with various membrane and insoluble fractions (Smith and Walker 1996). These enzymes are known to regulate phosphoenolpyruvate carboxylase, sucrose-phosphate synthase, nitrate reductase, 3-hydroxy-3-methylglutaryl-coenzymeA reductase (HMG-CoA), quinate dehydrogenase, and the plasma membrane H^+-ATPase (Smith and Walker 1996, Camoni et al. 2000, Dong et al. 2001).

Protein phosphatases-2B (PP2B) may regulate ion-channel activity in plant cells (Luan 2000).

Protein phosphatase-2C (PP2C) activity has been detected predominantly in the cytosol in carrot cells, cauliflower inflorescences, and leaves from peas and wheat (MacKintosh at al. 1991). They are present in the ABA signal transduction pathway, as a

partner protein for a receptor-like kinase. Three novel PP2C phosphatases have been cloned from *Arabidopsis*, but their physiological functions have yet to be elucidated (Smith and Walker 1996).

The protein tyrosine phosphatases (PTPase) family includes both tyrosine-specific and dual specificity phosphatases (Luan 2000). Unlike Ser/Thr phosphatases, PTPases appear to be abundant only in animal but not in yeast and plant systems (Luan 2000). However, the plant PTPases may function to regulate the MAPK pathway in response to a number of extracellular signals including high salts and cold (Luan 2000).

There are indications that other protein phosphatases are also present in plants: the mitochondrial pyruvate dehydrogenase phosphatase, chloroplast thylakoid protein phosphatases, protein tyrosine phosphatase, and probably many others (Smith and Walker 1996).

The above list of protein phosphatases is far from complete. Further studies will likely reveal the existence of other types of these important plant proteins.

1.10.3. Phosphorylation inhibitors

Inhibitors have been used extensively to study the involvement of protein phosphorylation in different cellular processes. There are a wide variety of chemical inhibitors more or less specific for a certain class of enzymes. Most of the studies carried out with chemical inhibitors examined the effect of these inhibitors on a particular protein or enzyme. There are only a few studies that have used these inhibitors to test the processes that take place at the whole organ or plant level. Their profound cellular effects

enhance our appreciation of the diversity of physiological processes that are controlled by reversible phosphorylation (MacKintosh and MacKintosh 1994). These inhibitors provide a very powerful approach for the initial assessment of the role of protein phosphorylation in controlling cellular events (Smith and Walker 1996). When used for *in vivo* studies, these inhibitors can provide only preliminary hints about the regulation of certain physiological processes. These studies have to be complemented with experiments at the molecular level. At the whole plant level due to the complexity of physiological processes, these inhibitors can provide preliminary insights on the regulation of a certain process, such as the root water uptake.

The effects of the following inhibitors were tested mostly against the mammalian enzymes. Nevertheless, these inhibitors may also be useful to understand the plant systems.

1.10.3.1. Phosphatase inhibitors

Okadaic acid is a major toxic compound associated with diarrhetic seafood poisoning (Cohen et al. 1990). It targets the serine-threonine phosphatases. The activity of PP2A was found to be completely inhibited at 1 nM concentration; PP1 was completely inhibited at 1 μM concentration; PP2B was inhibited by okadaic acid but with much lower potency (half maximal effect at 5 μM) (Cohen et al. 1990, Hardie et al. 1991, Smith and Walker 1996). PP2C, protein pyruvate dehydrogenase phosphatase, protein-tyrosine phosphatases, and acid and alkaline phosphatases were not inhibited by okadaic acid (Hardie et al. 1991).

Vanadium ions have been widely used to inhibit of protein-phosphotyrosine phosphatase (Gordon 1991). Vanadate is a well-known inhibitor of the plasma membrane associated ATPases and of phosphatases activities in plant cells (Hidalgo et al. 1988).

Fluoride can inhibit the activity of phosphoseryl and phosphothreonyl protein phosphatases (Struglics et al. 2000). It also decreases the activity of acid phosphatases (Zwiazek and Shay 1988) and the activity of plasma membrane H^+-ATPase (Rakowski et al. 1995).

1.10.3.2. Kinase inhibitors

6-dimethyl aminopurine (6-DMAP) was first recognized as a derivative of puromycin that was able to block cell division effectively in marine eggs (Rebhun et al. 1973). In general, 6-DMAP was found to inhibit a variety of animal protein kinases, and more recently, the tobacco protein kinase (Neant and Guerrier 1988, Mathieu et al. 1996). In tobacco cells, 6-DMAP is able to inhibit a part of protein phosphorylation occurring at the plasma membrane level (Mathieu et al. 1996).

Staurosporine was originally isolated as an antifungal alkaloid produced by *Streptomyces* sp. (Tamaoki et al. 1986). It is a potent inhibitor of protein kinases (Tamaoki 1991). Staurosporine is one of the most specific inhibitors of protein kinase C from mammals, but it can also inhibit PKA and PTK kinases (Tamaoki 1991, Yoshida and Anraku 2000). Staurosporine interacts at the ATP-binding site of protein kinases, inhibiting the activity of protein kinases (Hidaka and Kobayashi 1993).

1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) inhibits selectively either

protein kinase C or cyclic nucleotide dependent protein kinase (Hidaka et al. 1984). There are few reports that show the putative existence in plants of protein kinases similar to animal protein kinase C. Regarding the aquaporins, phosphorylation of PM28A in spinach leaves occurred on Ser-274, seven amino acids from the C terminus of the protein, within a consensus phosphorylarion site (Ser-X-Arg) for vertebrate protein kinase C (Johansson et. al. 1996).

1.10.3.3. Calmodulin inhibitors

When used in low concentration, **N-(6-Aminohexyl)-5-chloro-1-naphtalene-sulfoamide** (W-7) is a selective inhibitor of the calmodulin-dependent processes (Hidaka et al. 1991). However, compounds that inhibit calmodulin also interact with other protein targets beside calmodulin. W-7 can interact in a calcium-dependent manner with other calcium-modulated proteins (Roberts et al. 1986). Depending on the conditions of the experiment, calmodulin inhibitors may not distinguish between a calcium-calmodulin stimulated process and other calcium-dependent processes (Roberts et al. 1986).

Compounds like H-7 and W-7 compete with ATP. Therefore higher concentrations would be required to inhibit protein kinases when cells or tissues contain a large amount of ATP (Hidaka et. al. 1984). It is conceivable that when roots are metabolically depressed, they have lower concentrations of ATP, and smaller concentrations of these inhibitors can be effective.

1.11. Protein synthesis inhibitor

Cycloheximide is a glutarimide antibiotic that inhibits the synthesis of proteins encoded by nuclear genes. It affects both peptide initiation and extension by an effect on the donor ribosomal site (O'Brig et al. 1971).

1.12. Study objectives

The majority of aquaporins studies have been carried on herbaceous species and at the molecular level. Without doubt, these studies have revealed the molecular structure, function and regulation of the water channel protein. However, at the whole plant and organ levels, this regulation may be much more complex. For woody plants, due to their large dimensions and longevity, water transport is of particular importance. Water balance is also of major importance for the survival of seedlings after planting.

The mechanisms of aquaporin regulation are largely unknown. The role of aquaporins in regulating water fluxes across roots and at the whole plant level has started to become clear. So far, two major mechanisms for regulation of aquaporin activity have been proposed: 1) phosphorylation and dephosphorylation of aquaporins, and 2) regulation at the gene level. However, at the whole root system level, the contribution of those different mechanisms in modulating water fluxes across roots has not been extensively studied.

The present study investigated the mechanisms that may be used to regulate water flow properties in trembling aspen (*Populus tremuloides* Michx.) seedlings. Aspen is a

fast growing woody plant species and an important component of the boreal forest ecosystem.

The specific objectives of the present study were: 1) To investigate the regulation of root water uptake by phosphorylation and dephosphorylation events using inhibitors of protein kinases and phosphatases, and 2) To examine the importance of aquaporin synthesis in the process of root water flow regulation.

The general hypotheses of this study were: 1) Phosphorylation and dephosphorylation of aquaporins are the primary mechanisms regulating root water uptake, and 2) Aquaporin synthesis plays a secondary role in regulating root water uptake.

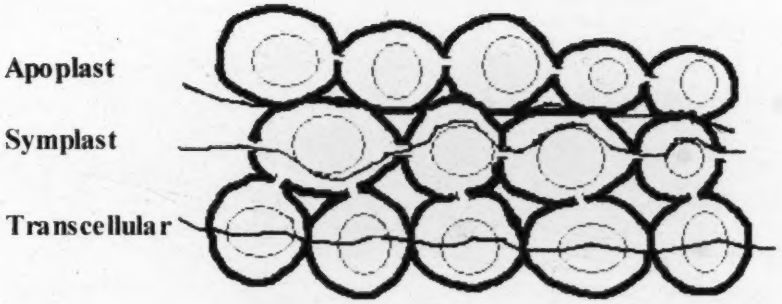


Figure 1.1 The three pathways for water movement into the roots: apoplast (through the cell walls and intercellular spaces), symplast (through the plasmodesmata and cytoplasm), and transcellular (across membranes). The symplastic and trancelluar pathways are considerend and referred together as the cell-to-cell pathway.

References

Agre, P., Preston, G.M., Smith, M.L., Jung, J.S., Raina, S., Moon, C., Guggino, W.B., and Nielsen, S. 1993. Aquaporin CHIP: the archetypal molecular water channel. Amer. J. Physiol. 265 (4 part 2): F463-F476.

Andersen, P.C., Lombard, P.B., and Westwood, M.N. 1984. Effect of anaerobiosis on the water relations of several *Pyrus* species. Physiol. Plant. 62: 245-252.

Azaizeh, H., and Steudle, E. 1991. Effects of salinity on water transport of excised maize (*Zea mays* L.) roots. Plant. Physiol. 97: 1136-1145.

Barford, D., Das, A.K., and Egloff, M.P. 1998. The structure and mechanism of protein phosphatases: Insight into catalysis and regulation. Ann. Rev. Biophys. Biomol. Struct. 27: 133-164.

Barone, L.M., Shih, C., and Wasserman, B.P. 1997. Mercury-induced conformational changes and identification of conserved surface loops in plasma membrane aquaporins from higher plants. J. Biol. Chem. 272(49): 30672-30677.

Barrieu, F., Chaumont, F., and Chrispeels, M.J. 1998. High expression of the tonoplast aquaporin ZmTIP1 in epidermal and conducting tissues of maize. Plant Physiol. 117: 1153-1163.

Barrieu, F., Marty-Mazars, D., Thomas, D., Chaumont, F., Charbonnier, M., and Marty, F. 1999. Dessication and osmotic stress increase the abundance of mRNA of the tonoplast aquaporin BobTIP26-1 in cauliflower cells. Planta 209: 77-86.

Barrowclough, D.E., Peterson, C.A., and Steudle, E. 2000. Radial conductivity along developing onion roots. J. Exp. Bot. 344: 547-557.

Beaudoin, N., Seriez, C., Gosti, F., and Giradaut, J. 2000. Interations between abscisic acid and ethylene signaling cascades. The Plant Cell 12: 1103-1115.

Becker, P., Tyree, M.T., and Tsuda, M. 1999. Hydraulic conductance of angiosperm versus conifers: similar transport sufficiency at the whole-plant level. Tree Physiol. 19: 445-452.

Bigot, J., and Boucad, J. 1994. Low-temperature pretreatment of the root system of *Brassica rapa* L. plants: effects on the xylem sap exudation and on the nitrate absorption rate. Plant Cell Environ. 17: 721-729.

Bolger, T.P., Upchurch, D.R., and McMichael, B.L. 1992. Temperature effects on cotton root hydraulic conductance. Environ. Exp. Bot. 32(1): 49-54.

Bradford, K.J., and Hsiao, T.C. 1982. Stomatal behaviour and water relations of waterlogged tomato plants. Plant Physiol. 70: 1508-1513.

Bush, D.S. 1995. Calcium regulation in plant cells and its role in signaling. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46: 95-122.

Byrne, G.F., Begg, J.E., and Hansen, G.K. 1977. Cavitation and resistance to water flow in plant roots. Agr. Meteor. 18: 21-25.

Camoni, L., Iori, V., Marras, M., and Adduci, P. 2000. Phosphorylation-dependent interaction between plant plasma membrane H^+-ATPase and 14-3-3 proteins. J. Biol. Chem. 275(14): 9919-9923.

Cantrill, L.C., Overall, R.L., and Goodwin, P.B. 1999. Cell-to-cell communication via plant endomembranes. Cell Biol. Intern. 23(10): 653-661.

Carvajal, M., Cerdá, A., and Martinez, V. 2000. Does calcium ameliorate the negative effect of NaCl on melon root water transport by regulationg aquaporin activity? New Phytol. 145: 439-447.

Carvajal, M., Cooke, D.T., and Clarkson, D.T. 1996. Responses of wheat plants to nutrient deprivation may involve the regulation of water-channel function. Planta 199: 372-381.

Carvajal, M., Cooke, D.T., and Clarkson, D.T. 1998. The lipid bilayer and aquaporins: parallel pathways for water movement into plant cells. Plant Growth Reg. 25: 89-95.

Carvajal, M., Martinez, V., and Alcaraz, C.F. 1999. Physiological function of water channels as affected by salinity in roots of paprika pepper. Physiol. Plant. 105: 95-101.

Chaumont, F., Barrieu, F., Jung, R., and Chrispeels, M.J. 2000. Plasma membrane intrinsic proteins from maize cluster in two sequence subgroups with differential aquaporin activity. Plant Physiol. 122: 1025-1034.

Chrispeels, M.J., and Agre, P. 1994. Aquaporins: water channel proteins of plant and animal cells. TIBS 19: 421-425.

Chrispeels, M., Crawford, N.M., and Schroeder, J.I. 1999. Proteins for transport of water and mineral nutrients across the membrane of plant cells. Plant Cell 11: 661-675.

Chrispeels, M.J., and Maurel, C. 1994. Aquaporins: the molecular basis of facilitated water movement through living plant cells? Plant Physiol. 105: 9-13.

Ciavatta, V.T., Morillon, R., Pullman, G.S., Chrispeels, M., and Cairney, J., 2001. An aquaglyceroporin is abundantly expressed early in the development of the suspensor and the embryo proper of loblolly pine. Plant Physiol. 127: 1556-1567.

Clarkson, D.T., Carvajal, M., Henzler, T., Waterhouse, R.N., Smyth, A.J., Cooke, D.T., and Steudle, E. 2000. Root hydraulic conductance: diurnal aquaporin expression and the effects of nutrient stress. J. Exp. Bot. 51(342): 61-70.

Cohen, P.T.W. 1997. Novel protein serine/threonine phosphatases: variety is the spice of life. TIBS 22: 245-251.

Cohen, P., Holmes, C.F.B., and Tsukitani, Y. 1990. Okadaic acid: a new probe for the study of cellular regulation. TIBS 15: 98-102.

Cramer, G.R., and Jones, R.L. 1996. Osmotic stress and abscisic acid reduce cytosolic calcium activities in roots of *Arabidopsis thaliana*. Plant Cell Environ. 19: 1291-1298.

Daniels, M.J., Chaumont, F., Mirkov, T.E. and Chrispeels, M.J. 1996. Characterization of a new vacuolar membrane aquaporin sensitive to mercury at a unique site. Plant Cell 5: 587-599.

Daniels, M.J., Chrispeels, M., and Yeager, M. 1999. Projection structure of a plant vacuolar membrane aquaporin by electron cryo-crystallography. J. Mol. Biol. 294: 1337-1349.

Dong, L., Ermolova, N.V., and Chollet, R. 2001. Partial purification and biochemical characterization of a heteromeric protein phosphatase 2A holoenzyme from maize (*Zea mays* L.) leaves that dephosphorylates C4 phosphoenolpyruvate carboxylase. Planta 213: 379-389.

Dordas, C., and Brown, P.H. 2001. Evidence for channel mediated transport of boric acid in squash (*Cucurbita pepo*). Plant Soil 235: 95-103.

Doussan, C., Pagès, L., and Vercambre, G. 1998a. Modelling of the hydraulic architecture of root system: an integrated approach to water absorbtion - model description. Ann. Bot. 81: 213-223.

Doussan, C., Vercambre, G., and Pagès, L. 1998b. Modelling of the hydraulic architecture of root system: an integrated approach to water absorbtion-distribution of axial conductances in maize. Ann. Bot. 81: 225-232.

Engel, A., Fujiyoshi, Y., and Agre, P. 2000. The importance of aquaporin water channel protein structures. The EMBO J. 19(5): 800-806.

Eckert, M., Biela, A., Siefritz, F., and Kaldenhoff, R. 1999. New aspects of plant aquaporin and specificity. J. Exp. Bot. 50: 1541-1545.

Else, M.A., Davies, W.J., Malone, M., and Jackson, M.B. 1995. A negative hydraulic message from oxygen-deficient roots of tomato plants? Plant Physiol. 109: 1017-1024.

Faiz, S.M.A., and Weatherley, P.E. 1978. Further investigation into the location and magnitude of the hydraulic resistances in the soil: plant system. New Phytol. 81: 19-28.

Fettiplace, R., and Haydon, D.A. 1980. Water permeability of lipid membranes. Physiol. Rev. 60(2): 510-550.

Finkelstein, A. 1987. Water movement through lipid bilayers, pores, and plasma membranes: theory and reality. John Wiley & Sons, Inc. New York.

Fiscus, E.L. 1975. The interaction between osmotic and pressure-induced water flow in plant roots. Plant. Physiol. 55: 917-922.

Fotidias, D., Jenö, P., Mini, T., Wirtz, S., Müller, S.A., Fraysse, L., Kjelbom, P., and Engel, A. 2001. Structural characterization of two aquaporins isolated from native spinach leaf plasma membrane. J. Biol. Chem. 276 (3): 1707-1714.

Frangne, N., Maeshima, M., Schäffner, A.R., Mandel, T., Martinoia, E., and Bonnemain, J.L. 2001. Expression and distribution of a vacuolar aquaporin in young and mature leaf tissues of *Brassica napus* in relation to water fluxes. Planta 212: 270-278.

Frensch, J., and Steudle, E. 1989. Axial and radial hydraulic resistance to roots of maize (*Zea mays* L.). Plant Physiol. 91: 719-726.

Freundl, E., Steudl, E., and Hartung, W. 2000. Apoplastic transport of abscisic acid through roots of maize: effect of the exodermis. Planta 210: 222-231.

Fricke, W. 2000. Water movement between epidermal cells of barley leaves - a symplastic connection? Plant Cell Environ. 23: 991-997.

Fu, D., Libson, A., Miercke, L.J.W., Weitzman, C., Nollert, P., Krucinski, J., Stroud, R.M. 2000. Structure

of a glycerol-conducting channel and the basis for its selectivity. Sci. 290: 481-486.

Gaspar, M., Sissoëff, I., Bousser, A., Roche, O., Mahé, A., and Hoarau, J. 2001. Transient variations of water transfer induced by HgCl2 in excised roots of young maize plants: new data on the inhibition process. Aust. J. Plant Physiol. 28: 1175-1186.

Gerbeau, P., Amodeo, G., Hnezler, T., Santoni, V., Ripoche, P. and Maurel, C. 2002. The water permeability of *Arabidopsis* plasma membrane is regulated by divalent cations and pH. Plant J. 30(1): 71-81.

Gerbeau, P., Güçlü, J., Ripoche, P., and Maurel, C. 1999. Aquaporin Nt-TIPa can account for the high permeability of tobacco cell vacuolar membrane to small neutral solutes. Plant J. 18(6): 577-587.

Gibbs, J., Turner, D.W., Armstrong, W., Darwent, M.J., and Greenway, H. 1998. Response to oxygen deficiency in the stele I-II. Development of oxygen deficiency in the stele reduces radial solute transport to the xylem. Aust. J. Plant Physiol. 25: 745-763.

Gordon, J.A. 1991. Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. Methods Enzym. 201: 477-482.

Gunsé, B., Poschenrieder, C., and Barceló, J. 1997. Water transport properties of roots and root cortical cells in proton- and Al-sensitive maize varieties. Plant Physiol. 113: 595-602.

Hakman, I., and Oliviusson, P. 2002. High expression of putative aquaporin genes in cells with transporting and nutritive functions during seed development in Norway spruce (*Picea abies*). J. Exp. Bot. 53(369): 639-649.

Hale, M.G., and Orcutt, D.M. 1987. The physiology of plants under stress: abiotic factors. John Wiley & Sons, Inc. New-York.

Hardie, D.G. 1999. Plant protein serine/threonine kinases: classification and functions. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 97-131.

Hardie, D.G. 2000. Plant protein-serine/threonine kinases: classification into subfamilies and overview of functions. In Plant protein kinases: advances in botanical research incorporating advances in plant pathology, edited by Kreis, M., Walker, J.C. & Callow, J.A. Academic Press, London, pp. 1-44.

Hardie, D.G., Haystead, T.A.J., and Sim, A.T.R. 1991. Use of okadaic acid to inhibit protein phosphatases in intact cells. Methods Enzym. 201: 469-482.

Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.S., Han, B., Zhu, T., Wang, X., Kreps, J.A., and Kay, S.A. 2000. Orchestrated trascription of key pathways in *Arabidopsis* by the circadian clock. Sci. 290: 2110-2113.

Harrington, C. 1987. Response of red alder and black cottonwood seedlings to flooding. Physiol. Plant. 69: 35-48.

Harvengt, P., Vlerick, A., Fuks, B., Wattiez, R., Ruysschaert, J.M., and Homble, F. 2000. Lentil seed aquaporins form a hetero-oligomer which is phosphorylated by a Mg^{2+}-dependent and Ca^{2+}-regulated kinase. Biochem. J. 352: 183-190.

Hejnowics, Z., and Sievers, A. 1996. Reversible closure of water channels in parenchymatic cells of

sunflower hypocotyl depends on turgour status of the cells. J. Plant Physiol. 147: 516-520.

Henzler, T., and Steudle, E. 1995. Reversible closing of water channels in *Chara* internodes provides evidence for a composite transport model of the plasma membrane. J. Exp. Bot. 46: 199-209.

Henzler, T., and Steudle, E. 2000. Transport and metabolic degradation of hydrogen peroxide in *Chara corallina*: a model calculations and measurement with the pressure probe suggest transport of H_2O_2 across water channels. J. Exp. Bot. 51(353): 2053-2066.

Henzler, T., Waterhouse, R.N., Smyth, A.J., Carvajal, M., Cooke, D.T., Schäffner, A.R., Steudle, E., and Clarkson, D.T. 1999. Diurnal variations in hydraulic conductivity and root pressure can be correlated with the expression of putative aquaporins in the root of Lotus japonicus. Planta 210: 50-60.

Heymann, J.B., and Engel, A. 1999. Aquaporins: Phylogeny, structure, and physiology of water channels. News Physiol. Sci. 14: 187-193.

Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. 1984. Isoquinolinesulfoamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochem. 23: 5036-5041.

Hidaka, H., and Kobayashi, R. 1993. Use of protein (serine/threonine) kinase activators and inhibitora to study protein phosphorylation in intact cells. In DG Hardie Eds. Protein posphorylation, a practical approach. Oxford University Press, Oxford, UK, pp. 87-107.

Hidaka, H., Watanabe, M., and Kabayasho, R., 1991. Properties and use of H-series compounds as protein kinase inhibitors. Methods Enzym. 201: 328-338.

Hidalgo, A., Navas, P., and Garcia-Herdugo, G. 1988. Growth inhibition induced by vanadate in onion roots. Environ. Exp. Bot. 28(2): 131-136.

Höfte, H., Hubbard, L., Reizer, J., Ludevid, D., Herman, E.M., and Chrispeels, M.J. 1992. Vegetative and seed-specific forms of tonoplast intrinsic protein in tha vacuolar membrane of *Arabisopsis thaliana.* Plant Physiol. 99: 561-570.

Hohmann, S., Bill, R.M., Kayingo, G., and Prior, B.A. 2000. Microbial MIP channels. Trends Microbiol. 8: 33-38.

Hose, E., Steudle, E., and Hartung, W. 2000. Abscisic acid and hydraulic conductivity of maize roots: a study using cell- and root-pressure probes. Planta 211: 874-882.

Hrabak, E.M. 2000. Calcium-dependent protein kinases and their relatives. In M Kreis, JC Walker & JA Callow Eds. Plant protein kinases: advances in botanical research incorporating advances in plant pathology. Academic Press, London, pp. 185-223.

Huang, B. 2000. Linking hydraulic conductivity to anatomy in plants that vary in specific root lenght. J. Amer. Soc. Hort. Sci. 125(2): 260-264.

Huang, B., and Nobel, P.S. 1994. Root hydraulic conductivity and its components, with emphasis on desert succulents. Agron. J. 86: 767-774.

Hunter, T. 1987. A thousand of protein kinases. Cell 50: 823-829.

Hunter, T. 1995. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 80: 225-236.

Huttley, A.K., and Phillips, A.L. 1995. Gibberellin-regulated plant genes. Physiol. Plant. 95: 310-317.

Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T., and Shinozaki, K. 2000. Prorein phosphorylation and dephosphorylation in environmental stress responses in plants. In M Kreis, JC Walker & JA Callow Eds. Plant protein kinases: advances in botanical research incorporating advances in plant pathology. Academic Press, London, pp. 355-377.

Ikeda, S., Nasrallah, J.B., Dixit, R., Preiss, S., and Nasrallah, M.E. 1997. An aquaporin-like gene requires for the *Brassica* self-incompatibility response. Sci. 276: 1564-1566.

Ishibashi, K., and Sasaki, S. 1998. The dichotomy of MIP family suggests two separates origins of water channels. News Physiol. Sci. 13: 137-142.

Jackson, R.B., Sperry, J.S., and Dawson, T.E. 2000. Root water uptake and transport: using physiological processes in global prediction. Trend Plant Sci. 5(11): 482-488.

Jackson, M.B., Davies, W.J., and Else, M.A. 1996. Pressure-flow relationship, xylem solutes and root hydraulic conductance in flooded tomato plants. Ann. Bot. 77: 17-24.

Javot, H., and Maurel, C. 2002. The role of aquaporins in root water uptake. Annals of Botany 90: 301-313.

Johansson, I., Karlsson, M., Johansson, U., Larsson, C., and Kjelbom, P. 2000. The role of aquaporins in cellular and whole plant water balance. Biochim. Biophys. Acta 1465: 324-342.

Johansson, I., Karlsson, M., Shukla, V.K., Chrispeels, M.J., Larsson, C., and Kjellbom, P. 1998. Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. Plant Cell 10: 451-460.

Johansson, I., Larsson, C., Ek, B., and Kjellbom, P. 1996. The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^{2+} and apoplastic water potential. The Plant Cell 8: 1181-1191.

Jouannic, S., Leprince, A.-S., Hamal, A., Picaud, A., Kreis, M., and Henry, Y. 2000. Plant mitogen-activated protein kinase signalling pathways in the limelight. In M Kreis, JC Walker & JA Callow Eds. Plant protein kinases: advances in botanical research incorporating advances in plant pathology. Academic Press, London, pp. 299-354.

Jung, J.S., Preston, G.M., Smith, B.L., Guggino, W.B., and Agre, P. 1994. Molecular structure of the water channel through aquaporin CHIP. The hourglass model. J. Biol. Chem. 269(20): 14658-14654.

Kaldenhoff, R., Grote, K., Zhu, J.J., and Zimmermann, U. 1998. Significance of plasmalemma aquaporins for water transport in *Arabidopsis thaliana*. Plant J. 14(1): 121-128.

Kamaluddin, M., and Zwiazek, J.J. 2001. Metabolic inhibition of root water flow in red-osier dogwood (*Cornus stolonifera*) seedlings. J. Exp. Bot. 52 (357): 739-745.

Kamaluddin, M., and Zwiazek, J.J. 2002. Ethylene enhances water transport in hypoxic aspen. Plant Physiol. 128: 1-8.

Karlsson, M., Johansson, I., Bush, M., McCann, M.C., Maurel, C., Larsson, C., and Kjellbom, P. 2000. An

abundant TIP expressed in mature highly vacuolated cells. Plant J. 21(1): 83-90.

Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D., and Bohnert, H.J. 2001. Gene espression profiles during the initial phase of salt stress in rice. The Plant Cell 13: 889-905.

Kikuyama, M., and Tazawa, M., 1998. Involvement of water channels in the hydration-induced calcium release in *Nitella flexis*. Plant Cell Physiol. 39(8): 830-835.

Kimmins, J.P. 1996. Forest Ecology 2nd ed. Prentice-Hall, New Jersey, USA.

King, L.S., Yasui, M., and Agre, P. 2000. Aquaporins in health and disease. Molec. Med.Today 6: 60-65.

Kirch, H.H., Vera-Estrella, R., Golldack, D., Quigley, F., Michalowski, C.B., Barkla, B.J., and Bohnert, H.J. 2000. Expression of water channel proteins in *Messembryanthemum crystallinum*. Plant Physiol. 123: 111-124.

Kjelbom, P., Larsson, C., Johansson, I., Karlsson, M., and Johanson, U. 1999. Aquaporins and water homeostasis in plants. Trends Plant Sci. 4(8): 308-314.

Lambers, H., Chapin III, F.S., and Pons, T.L. 1998. Plant physiological ecology. Springer-Verlag, New York.

Levitt, J. 1980. Responses of plants to environmental stresses. 2nd ed. vol. II, Academic Press, New York.

Levy, Y., and Syvertsen, J.P. 1983. Effect of drought stress and vesicular-arbuscular mycorrhiza on citrus transpiration and hydraulic conductivity of roots. New Phytol. 93: 61-66.

Lo Gullo, M.A., Nardini, A., Salleo, S., and Tyree, M.T. 1998. Changes in root hydraulic conductance (KR) of *Olea oleaster* seedlings following drought stress and irrigation. New Phytol. 140: 25-31.

Lopez, O.R., and Kursar, T.A. 1999. Flood tolerance of four tropical species. Tree Physiol. 19: 925-932.

Lu, Z., and Neumann, P.M. 1999. Water stress inhibits hydraulic conductance and leaf growth in rice seedlings but not the transport via mercury-sensitive water channels in the root. Plant Physiol. 120: 143-151.

Luan, S. 2000. Protein phosphatases: structure, regulation, and function. In M Kreis, JC Walker & JA Callow Eds. Plant protein kinases: advances in botanical research incorporating advances in plant pathology. Academic Press, London, pp. 67-107.

Ludevid, D., Höfte, H., Himelblau, E., and Chrispeels, M.J. 1992. The expression pattern of the tonoplast intrinsic protein γ-TIP in *Arabidopsis thaliana* is correlated with cell enlargement. Plant. Physiol. 100: 1633-1639.

Ma, T., Frigeri, A., Tsai, S.-T., Verbavats, J.-M., and Verkman, A.S. 1993. Localization and functional analysis of CHIP28k water channels in stably transfected chinese hamster ovary cells. J. Biol. Chem. 268(30): 22756-22764.

MacKintosh, C., and MacKintosh, R.W. 1994. Inhibitors of protein kinases and phosphatases. TIBS 19: 444-448.

MacKintosh, C., Coggins, J., and Cohen, P. 1991. Plant protein phosphatases. Subcellular distribution, detection of protein phosphatase 2C and identification of protein phosphatase 2A as the major

quinate dehydrogenase phosphatase. Biochem. J. 273: 733-738.

Maggio, A., and Joly, R.J. 1995. Effects of mercuric chloride on the conductivity of tomato root system. Plant Physiol. 109: 331-335.

Marin-Olivier, M., Chevalier, T., Fobis-Loisy, I., Dumas, C., and Gaude, T. 2000. Aquaporin PIP genes are not expressed in the stigma papillae in *Brassica oleracea*. Plant J. 24(2): 231-240.

Markhart, A.H. 1984. Amelioration of chilling-induced water stress by abscisic acid-induced changes in root hydraulic conductance. Plant Physiol. 74: 81-83.

Markhart, A.H., and Smit, B. 1990. Measurement of root hydraulic conductance. HortSci. 25: 282-287.

Martinez-Ballesta, M.C., Martinez, V., and Carvajal, M. 2000. Regulation of water channel activity in whole roots and in protoplasts from roots of melon plants grown under saline conditions. Aust. J. Plant Physiol. 27: 685-691.

Mathieu, Y., Snachez, F.J., Droillard, M.-J., Lapous, D., Laurière, C., and Guern, J. 1996. Involvement of protein phophorylation in the early steps of transduction of the oligogalacturonide signal in tobacco cell. Plant Physiol. Biochem. 34(3): 399-408.

Maurel, C. 1997. Aquporins and water permeability of plant membranes. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 399-420.

Maurel, C., Chrispeels, M.J., Lurin, C., Tacnet, F., Geelen, D., Ripoche, P., and Guern, J. 1997. Function and regulation of seed aquaporins. J. Exp. Bot. 48(Special Issue): 421-430.

Maurel, C., and Chrispeels, M.J. 2001. Aquaporins. A molecular entry into plant water relations. Plant Physiol. 125: 135-138.

Maurel, C., Kado, R.T., Guern, J., and Chrispeels, M.J. 1995. Phosphorylation regulates the water channel activity of the seed-specific aquaporin α-TIP. The EMBO J. 14(13): 3028-3035.

Morillon, R., Catterou, M., Sangwan, R.S., Sangwan, B.S., and Lassalles, J.P. 2001. Brassinolide may control aquaporin activities in *Arabidopsis thaliana*. Planta 212: 199-204.

Morillon, R., and Chrispeels, M. 2001. The role of ABA and the trasnpiration stream in the regulation of the osmotic water permeability of leaf cells. Proc. Natl. Acad. Sci. USA 98(24): 14138-14143.

Morillon, R., and Lassalles, J.-P. 2002. Water deficit during root development: effects on the growth of roots and osmotic water permeability of isolated root protoplasts. Planta 214: 392-399.

Moshelion, M., Becker, D., Biela, A., Uehlein, N., Hedrich, R., Otto, B., Levi, H., Moran, N., and Kaldenhoff, R. 2002. Plasma membrane aquaporins in the motor cells of *Samanea saman*: diurnal and circadian regulation. Plant Cell 14: 727-739.

Murphy, R., and Smith, J.A.C. 1998. Determination of cell water-relations parameters using the pressure probe: extended theory and practice of the pressure-clamp technique. Plant Cell Environ. 21: 637-657.

Nardini, A., and Pitt, F. 1999. Drought resistance of *Quercus pubescens* as a function of root hydraulic conductance, xylem embolism and hydraulic architecture. New Phytol. 143: 485-493.

Nardini, A., Salleo, S., and Lo Gullo, M.A. 1998. Root hydraulic conductance of six forest trees: possible

adaptive significance of seasonal changes. Plant Biosyst. 132: 97-104.

Neant, I., and Guerrier, P., 1988. 6-Dimethylaminopurine blocks starfish oocyte maturation by inhibiting a relevant protein kinase activity. Exp. Cell Research 176: 68-79.

Németh-Cahalan, K., and Hall, J.K. 2000. pH and calcium regulate the water permeability of aquaporin 0. J. Biol. Chem. 275(10): 6777-6782.

Netting, A.G. 2000. pH, abscisic acid and the integration of metabolism in plants under stressed and non-stressed conditions. I Cellular responses to stress and their implication for plant water relations. J. Exp. Bot. 51(343): 147-158.

Nilsen, E.T., and Orcutt, D.M. 1996. Physiology of plants under stress. Abiotic factors. John Wiley & Sons, Inc. New York.

Nielsen, S., and Agre, P. 1995. The aquaporin family of water channels in kidney. Kidney Intern. 48: 1057-1068.

Niemitz, C.M., and Tyreman, S.D. 1997. Characterization of water channels in wheat root membrane vesicles. Plant Physiol. 115: 561-567.

Niemitz, C.M., and Tyreman, S.D. 2000. Channel-mediated permeation of ammonia gas through the peribacteroid membrane of soybean nodules. FEBS Letters 465: 110-114.

Nobel, P.S., and Lee, C.H. 1991. Variations in root water potentials: influences of of environmental factors for two succulent species. Ann. Bot. 67: 549-554.

Nobel, P.S., Schulte, P.J., and North, G.B. 1990. Water influx characteristics and hydraulic conductivity for roots of *Agave deserti* Engelm. J. Exp. Bot. 225: 409-415.

North, G.B., and Nobel, P.S. 1995. Hydraulic conductivity of concentric root tissues of *Agave deserti* Engelm. under wet and drying conditions. New Phytol. 130: 47-57.

North, G.B., and Nobel, P.S. 1996. Radial hydraulic conductivity of individual root tissues of *Opuntia ficus-indica* (L.) Miller as soil moisture varies. Ann. Bot. 77: 133-142.

O'Brig, T.G., Culp, W.J., McKeenan, W.L., and Boyd, H. 1971. The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis in reticulocyte ribosomes. J. Biol. Chem. 246(1): 174-181.

Ohshima, Y., iwasaki, I., Suga, S., Murakami, M., Inoue, K., and Maeshima, M. 2001. Low aquaporin content and low osmotic permeability of the plasma and vacuolar membranes of a CAM plant *Graptopetalum paraguayense*: comparison with radish. Plant Cell Physiol. 42(10): 1119-1129.

Otto, B., and Kaldenhoff, R. 2000. Cell-specific expression of the mercury-insensitive plasma-membrane aquaporin NtAQP1 from *Nicotiana tabacum*. Planta 211: 167-172.

Parson, L.R., and Kramer, P.J. 1974. Diurnal cycling in root resistance to water movement. Physiol. Plant. 30: 19-23.

Passioura, J.B. 1988. Water transport in and to roots. Ann. Rev. Plant. Physiol. Plant Mol. Biol. 34: 245-265.

Peterson, C.A., Murrmann, M., and Steudle, E. 1993. Location of the major barriers to water and ion

movement in young roots of *Zea mays* L. Planta 190: 127-136.

Poovaiah, B.W., and Reddy, A.S.N. 1987. Calcium messenger system in plants. CRC Critic. Rev. Plant Sci. 6(1): 47-102.

Qiu, Q.S., Wang, Z.Z., Zhang, N., Cai, Q.G., and Jiang, R.X. 2000. Aquaporins in the plasma membrane of leaf callus protoplasts of *Acnitia deliciosa* var. *deliciosa* cv. Hayward. Aust. J. Plant Physiol. 27: 71-75.

Quintero, J.M., Fournier, J.M., and Benlloch, M. 1999. Water transport in sunflower root systems: effects of ABA, Ca^{2+} status and $HgCl_2$. J. Exp. Bot. 50 (339): 1607-1612.

Rakowski, K.J., and Zwiazek, J.J., and Sumner, M.J. 1995. Hydrogen fluoride effects on plasma membrane composition, ATPase activity and cell structure in needles of eastern white pine (*Pinus strobus*) seedlings. Trees 9: 190-194.

Rebhun, L.I., White, D., Sander, G., and Ivy, N. 1973. Cleavage inhibition in marine eggs by puromycin and 6-dimethylaminopurine. Exp. Cell Res. 77: 312-318.

Reece, C.F., and Riha, S.J. 1991. Role of root system of eastern larch and white spruce in response to flooding. Plant Cell Environ. 14: 229-234.

Ren, G., Cheng, A., Melnyk, P., and Mitra, A.K. 2000. Polymorphism in the paking of aquaporin-1 tetramers in 2-D crystals. J. Struc. Biol. 130: 45-53.

Rieger, M., and Litvin, P. 1999. Root system hydraulic conductivity in species with contrasting root anatomy. J. Exp. Bot. 331: 201-209.

Roberts, D.M., and Harmon, A.C. 1992. Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43: 375-414.

Roberts, D.M., Lukas, T.J., and Watterson, D.M. 1986. Structure, function, and mechanism of action of calmodulin. CRC Critic. Rev. Plant Sci. 4(4): 311-339.

Robinson, D.G., Sieber, H., Kammerloher, W., and Schäffner, A.R. 1996. PIP1 aquaporins are concentrates in plasmalemmasomes of *Arabidopsis thaliana* mesophyll. Plant Physiol. 111: 645-649.

Rygol, J., Arnold, W.M., and Zimmermann, U. 1992. Zinc and salinity effects on membrane transport in *Chara connivens*. Plant Cell Environ. 15: 11-23.

Saliendra, N.Z., and Meinzer, F.C. 1992. Genotypic, developmental and drought-induced differences in root hydraulic conductance of contrasting sugarcane cultivars. J. Exp. Bot. 254: 1209-1217.

Sanders, D., Brownlee, C., and Harper, J.F. 1999. Communicating with calcium. Plant Cell 11: 691-706.

Schäffner, A.R. 1998. Aquaporin function, structure, and expression: are there more surprises to surface in water relation? Planta 204(2): 131-139.

Schmull, M., and Thomas, F.M. 2000. Morphological and physiological reactions of young deciduous trees (*Quercus robur* L., *Q. petraea* (Matt.) Liebl., *Fagus sylvatica* L.) to waterlogging. Plant Soil 225: 227-242.

Sena-Gomes, A.R., and Kozlowski, T.T. 1980. Growth responses and adaptation of *Fraxinus pennsylvanica* seedlings to flooding. Plant Physiol. 66: 267-271.

Shenolikar, S. 1994. Protein serine/threonine phosphatases - New avenues for cell regulation. Annu. Rev. Cell. Biol. 10: 55-86.

Siefritz, F., Tyree, M.T., Lovisolo, C., Schubert, A., and Kaldenhoff, R. 2002. PIP1 plasma membrane aquaporins in tobacco: from cellular effects to function in plants. Plant Cell 14: 869-876.

Smart, L.B., Moskal, W.A., Cameron, K.D., and Bennett, A.B. 2001. MIP genes are down-regulated under drought stress in *Nicotiana glauca*. Plant Cell Physiol. 42(7): 686-693.

Smit, B., and Stachowiak, M. 1988. Effects of hypoxia and elevated carbon dioxide concentration on water flux through *Populus* roots. Tree Physiol. 4: 153-165.

Smith, R.D., and Walker, J.C. 1996. Plant protein phosphatases. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 101-125.

Steudle, E. 1993. Pressure probe techniques: basic principles and application to studies of water and solute relations at the cell, tissue, and organ level. In JAC Smith and H Griffith Eds. Water deficits: plant responses from cell to community. Bios Scientific Publisher, Oxford, pp. 5-36.

Steudle, E. 1994. Water transport across roots. Plant Soil 167: 79-90.

Steudle, E. 2000a. Water uptake by roots: effects of water deficit. J. Exp. Bot. 51(350): 1531-1542.

Steudle, E. 2000b. Water uptake by the plant roots: an integration of views. Plant Soil 226: 45-56.

Steudle, E. 2001. The cohesion-tension mechanism and the acquisition of water by plant roots. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52: 847-875.

Steudle, E., and Frensch, J. 1996. Water transport in plants: role of the apoplast. Plant Soil 187: 67-79.

Steudle, E., and Henzler, T. 1995. Water channels in plants: do basic concepts of water transport change? J. Exp. Bot. 46(290): 1067-1076.

Steudle, E., Murrmann, M., and Peterson, C.A. 1993. Transport of water and solutes across maize roots modified by puncturing the endodermis. Further evidence for the composite transport model of the root. Plant Physiol. 103: 335-349.

Steudle, E., and Peterson, C.A. 1998. How does water get through roots? J. Exp. Bot. 49(322): 775-778.

Steudle, E., and Tyreman, S.D. 1983. Determination of permeability coefficients, reflection coefficients, and hydraulic conductivity of *Chara corallina* using the pressure probe: effects of solute concentration. J. Membrane Biol. 75: 85-96.

Steudle, E., Zimmermann, U., and Zillikens, J. 1982. Effect of cell turgor on hydraulic conductivity and elastic modulus of *Elodea* leaf cells. Planta 154: 371-380.

Stone, J.M., and Walker, J.C. 1995. Plant protein kinase families and signal transduction. Plant Physiol. 108: 451-457.

Struglics, A., Fredlund, K.M., Kostantinov, Y.M., Allen, J.F., and Møller, I.M. 2000. Protein phosphorylation/dephosphorylation in the inner membrane of potato tuber mitochondria. FEBS Letters 475: 213-217.

Suga, S., Imagawa, S., and Maeshima, M. 2001. Specificity of the accumulation of mRNAs and proteins of the plasma membrane and tonoplast aquaporins in radish organs. Planta 212: 294-304.

Suwastika, I.N., and Gehring, C.A. 1998. Natriuretic peptides hormones promote radial water movements from the xylem of *Tradescantia* shoots. Cell. Mol. Life Sci. 54: 1161-1167.

Tamaoki, T. 1991. Use and specificity of Staurosporine, UNC-01, and calphosptin C as protein kinase inhibitors. Methods Enzym. 201: 340-347.

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F. 1986. Stauroporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. Biochem. Biophys. Res. Comm. 135(2): 397-402.

Taylor, H.M., and Willat, T. 1983. Shrinkage of soybean roots. Agron. J. 75: 818-820.

Tazawa, M., Asai, K., and Iwasaki, N. 1996. Characteristics of Hg- and Zn-sensitive water channels in the plasma membrane of *Chara* cells. Bot. Acta 109: 388-396.

Tazawa, M., Sutou, E., and Shibasaka, M. 2001. Onion root water transport sensitive to water channel and K+ channel inhibitor. Plant Cell Physiol. 42(1): 28-36.

Tchernov, D., Helman, Y., Keren, N., Luz., B., Ohad, I., Reinhold, L., Ogawas, T., and Kaplan, A. 2001. Passive entry of CO_2 and its energy-dependent intracellular conversion to HCO_3^- in cyanobacteria are driven by a photosystem I-generated $\Delta\mu H^+$. J. Biol. Chem. 276(26): 23450-23455.

Torii, K.U., and Clark, S.E. 2000. Receptor-like kinases in plant development. In M Kreis, JC Walker & JA Callow Eds. Plant protein kinases: advances in botanical research incorporating advances in plant pathology. Academic Press, London, pp. 226-267.

Tyree, M.T., and Ewers, F.W. 1991. The hydraulic architecture of trees and other woody plants. New Phytol. 119: 345-360.

Tyree, M.T., Patiño, S., Bennink, J., and Alexander, J. 1995. Dynamic measurements of root hydraulic conductance using a high-pressure flowmeter in the laboratory and field. J. Exp. Bot. 46: 83-94.

Tyreman, S.D., Bohnert, H.J., Maurel, C., Steudle, E., and Smith, J.A.C. 1999. Plant aquaporins: their molecular biology, biophysics and significance for plant water relations. J. Exp. Bot. 50: 1055-1071.

Tyreman, S.D., Niemietz, C.M., and Bramley, H. 2002. Plant aquaporins: multifunctional water and solute chanls with expanding roles. Plant Cell Environ. 25: 173-194.

Vera-Estrella, R., Barkla, B.J., Jiggins, V.J., and Blumwald, E. 1994. Plant defense response to fungal pathogens: activation of host-plasma membrane H^+-ATPase by elicitor-induced enzyme dephosphorylation. Plant Physiol. 104: 209-215.

Wan, X., Landhäusser, S.M., Zwiazek, J.J., and Lieffers, V.J. 1999. Root water flow and growth of aspen (*Populus tremuloides*) at low root temperatures. Tree Physiology 19: 879-884.

Wan, X., and Zwiazek, J.J. 1999.Mercuric chloride effects on root water transport in aspen seedlings. Plant Physiol. 121: 939-946.

Wan, X., and Zwiazek, J.J. 2001. Root water flow and leaf stomatal conductance in aspen (*Populus tremuloides*) seedlings treated with abscisic acid. Planta 213: 741-747.

Wan, X., Zwiazek, J.J., Lieffers, V.J., and Landhäusser, S.M. 2001. Hydraulic conductance in aspen (*Populus tremuloides*) seedlings exposed to low root temperatures. Tree Physiology 21: 691-696.

Wang, Y.H., Garvin, D.F., and Kochian, L.V. 2001. Nitrate-induced genes in tomato roots. Array analysis reveals novel genes that may play a role in nitrogen nutrition. Plant Physiol. 127: 345-359.

Weaver, C.D., Crombie, B., Stacey, G., and Roberts, D.M. 1991. Calcium-dependent phosphorylation of symbiosome membrane proteins from nitrogen-fixing soybean nodules. Plant Physiol. 95: 222-227.

Weig, A., Deswarte, C., and Chrispeels, M.J. 1997. The major intrinsic protein family of *Arabidopsis* has 23 members that form three distinct groups with functional aquaporins in each group. Plant Physiol. 114: 1347-1357.

Wilkinson, S., and Davies, W.J. 2002. ABA-based chemical signaling: the co-ordination of responses to stress in plants. Plant Cell Environ. 25: 195-210.

Woltering, E.J. 1990. Interorgan translocation of 1-aminocyclopropane-1-carboxylic acid and ethylene coordinates senescence in emasculated *Cymbidium* flowers. Plant Physiol. 92: 837-845.

Yamada, S., Nelson, D.E., Ley, E., Marquez, S., and Bohnert, H.J. 1997. The expression of an aquaporin promoter from *Mesembryanthemum crystallinum* in tobacco. Plant Cell Physiol. 38(12): 1326-1332.

Yoshida, S., and Anraku, Y. 2000. Characterization of staurosporine-sensitive mutants of *Saccharamyces cerevisiae*: vacuolar functions affect staurosporine sensitivity. Mol. Gen. Genet. 263: 877-888.

Zardoya, R., and Villalba, S. 2001. A phylogenetic framework for the aquaporin family in eukaryotes. J. Mol. Evol. 52: 391-404.

Zhang, J., Schurr, U., and Davies, W.J. 1987. Control of stomatal behaviour by abscisic acid which apparently originates in the roots. J. Exp. Bot. 38(192): 1174-1181.

Zhang, W.H., and Tyreman, S.D. 1991. Effect of low O_2 concentration on azide on hydraulic conductivity and osmotic volume of the cortical cells of wheat roots. Aust. J. Plant Physiol. 18: 603-613.

Zhang, W.H., and Tyreman, S.D. 1999. Inhibition of water channels by $HgCl_2$ in intact wheat root cells. Plant Physiol. 120: 849-857.

Zhu, G.L., and Steudle, E. 1991. Water transport across maize roots. Simultaneous measurement of flows at the cell and root level by double pressure probe technique. Plant Physiol. 95: 305-315.

Zimmermann, H.M., Hartmann, K., Schreiber, L., and Steudle, E. 2000. Chemical composition of apoplastic barriers in relation to radial hydraulic conductivity of corn roots (*Zea mays* L.). Planta 210: 302-311.

Zimmermann, H.M., and Steudle, E. 1998. Apoplastic transport across young maize roots: effects of the exodermis. Planta 206: 7-19.

Zwiazek, J.J., and Shay, J.M. 1988. Sodium fluoride induced metabolic changes in jack pine seedlings. II. Effects on growth, acid phosphatase, cytokinins, and pools of soluble proteins, amino acids, and organic acids. Can. J. For. Res. 18: 1311-1317.

Chapter 2. Effects of phosphorylation, dephosphorylation and calmodulin inhibitors on water flow properties in roots of aspen (*Populus tremuloides* Michx.) seedlings

2.1. Introduction

Efficient absorption of water and mineral nutrients by plants depends on the size of their root system and its water permeability. According to the composite model, water flow in roots occurs via three parallel pathways: apoplastic, symplastic, and transcellular (Steudle and Frensch 1996, Steudle 2000). The symplastic and transcellular pathways are usually considered together as the cell-to cell pathway (Steudle and Frensch 1996, Steudle 2000).

In the cell-to-cell pathway, fine regulation of water movement is mediated by water channel proteins, also referred to as the aquaporins (Steudle 2000). Reversible inhibition of root water uptake with $HgCl_2$ was obtained in roots of aspen seedlings (Wan and Zwiazek 1999), tomato (Maggio and Joly 1995), paprika pepper (Carvajal et al. 1999), and barley (Tazawa et al. 1997). These studies suggest that water channels participate in regulating root hydraulic conductivity and plant water balance.

The water transport activity of some aquaporins can be increased by reversible phosphorylation, which points to this process as a possible gating mechanism for aquaporin function. The phosphorylation of PM28A, the major integral protein of spinach leaf plasma membrane, is Ca^{2+}-dependent and involves the participation of Ca^{2+}-dependent protein kinases (CDPK) (Johansson et al. 1996, Johansson et al. 1998). The

phosphorylation of α-TIP aquaporin is carried out by a Mg^{2+}-dependent and Ca^{2+}-regulated membrane bound protein kinase, possibly belonging to the calcium-dependent protein kinases (CDPK) family (Harvengt et al. 2000). The increase in root hydraulic conductivity of hypoxic aspen roots exposed to ethylene was also thought to be mediated through the phosphorylation of water channel proteins in the root plasma membranes (Kamaluddin and Zwiazek 2002). However, no study has evaluated the contribution of phosphorylation and dephosphorylation events to the total root water transport.

Water channel proteins have been proposed to mediate the rapid adjustment of root water permeability in response to environmental factors (Javot and Maurel 2002). However, the elements that link the aquaporins with environmental factors are still missing. In plant cells, calcium, lipids, pH, and cyclic MP mediate the actions of many extracellular signals (Sanders at al. 1999). Some of the principal targets in Ca^{2+} signal transduction pathways are calmodulin and the calcium-dependent protein kinases (Sanders et al. 1999). An ameliorative effect of Ca^{2+} on root hydraulic conductivity (L_p) has been reported in salt-stressed roots (Martine-Ballestra et al. 2000, Carvajal et al. 2000), although Ca^{2+} can also directly block aquaporins (Gerbeau et al. 2002). Calmodulin inhibitors increased water permeability of the mammalian aquaporin AQP0, suggesting that Ca^{2+} may act through calmodulin (Németh-Cahalan and Hall 2000). The phosphorylation motif of the CDPK, the enzyme that phosphorylates the PM28A aquaporins in spinach leaves, overlaps with the known calmodulin-binding site (Huang et al. 2001).

Another important factor in the plant-environment interaction is the soil pH. Low pH has various effects on water permeability of cells with no effect (Gunsé et al. 1997),

decrease (Gerbeau at al. 2002), and increase in water permeability reported for the mammalian AQP0 (Németh-Cahalan and Hall 2000). The effect of high pH on water permeability in cells is less documented.

In the present study, I investigated the involvement of phosphorylation and dephosphorylation events and calmodulin in regulating water fluxes of the intact root systems in aspen (*Populus tremuloides* Michx.) seedlings grown in solution culture. Water transport in roots was studied using the pressure-flux approach (Markhart et al. 1979, Wan et al. 1999). Previous studies demonstrated that aquaporin activity is affected by root respiration (Zhang and Tyreman 1999, Kamaluddin and Zwiazek 2001). Therefore, in the present study, respiration was assessed in treated and control roots. The activity of root aquaporins was investigated using phosphorylation and dephosphorylation, and calmodulin inhibitors. Although in other studies, these inhibitors have been mostly used at the cellular level, they can also provide an initial assessment of the processes involved in water transport in roots.

In the present study I tested the following hypotheses: 1) Phosphorylation and dephosphorylation are the primary processes used to regulate the root water uptake; 2) Root respiration is not the primary regulatory mechanism of root water transport; 3) Regulation of root water uptake involves calmodulin-dependent processes.

This study was designed to confirm earlier results (Johansson et al. 1998, Harvengt et al. 2000, Kamaluddin and Zwiazek 2002, Kamaluddin and Zwiazek 2003) that suggested the importance of phosphorylation in aquaporin regulation in roots. As suggested by previous studies (Pedersen 1993, Fleurat-Lessard et al. 1997, Zhang and Tyreman 1999), water transport is energy dependent and, possibly, linked to proton

fluxes across the plasma membrane.

2.2. Materials and Methods

2.2.1. Plant material

Aspen (*Populus tremuloides* Michx.) seeds were collected from trees in Edmonton, Alberta, Canada. The seeds were germinated in sand and 5 days after germination, the seedlings were moved to individual 60 ml styroblock containers (Super Block, Beaver Plastic, Edmonton, AB). The containers were filled with mixture of sand, peat, and perlite (3:3:1, by volume). The seedlings were grown for 6 weeks in a growth room with a 16 h photoperiod, photosynthetic photon flux density of 300 μmol m^{-2} s^{-1}, 22°C/18°C (day/night) temperatures, and relative humidity of approximately 70%. During that time, the seedlings were bottom-watered with distilled water and fertilized twice a week with 0.1% 20-20-20 (N-P-K) commercial fertilizer solution. When 6-weeks old, the seedlings were moved to solution culture. The roots were gently washed free of soil and the seedlings were placed in 11 l plastic containers, with 10 seedlings per container. The containers were filled with full-strength modified Hoagland's mineral solution (Epstein 1972) and aerated with an air pump. To avoid hypoxic conditions, solutions were aerated and O_2 level monitored with a dissolved O_2 probe (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.). The dissolved O_2 concentration was approximately 7-8 mg l^{-1}. After two weeks, the seedlings were removed from the containers to measure root water flow, root respiration and acid phosphatase activity.

2.2.2. Chemical inhibitors

Several chemical inhibitors were used to study their effects on root water uptake. Based on their action within cells, the chemical inhibitors were grouped into phosphorylation inhibitors (protein kinase inhibitors), dephosphorylation inhibitors (protein phosphatase inhibitors), and calmodulin inhibitor. The phosphorylation inhibitors used in this study were: 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) (50 μM), staurosporine (0.7 μM), and 6-dimethyl aminopurine (6-DMAP) (1 mM and 5 mM) (Mathieu et al. 1996, Hidaka and Kobayashi 1993, Hidaka et al. 1984). The following dephosphorylation inhibitors were tested: sodium orthovanadate (Na_3VO_4) (1 mM and 5 mM), potassium fluoride (KF) (1 mM and 5 mM), okadaic acid free acid (OA) (0.2 μM) and okadaic acid ammonium salt (OA) (1 μM) (Hardie et al. 1991, Hidalgo et al. 1988, Struglics et al. 2000). A single calmodulin inhibitor was used: N-(6-Aminohexyl)-5-chloro-1-naphtalene-sulfoamide, W-7 (50 μM and 150 μM) (Hidaka et al. 1991). As sodium control for Na_3VO_4, 5 mM sodium orthophosphate (Na_3PO_4 x 12 H_2O) was used. Sodium orthovanadate, KF, okadaic acid free acid, H-7, 6-DMAP, and W-7 were purchased from Sigma-Aldrich (Oakville, ON, Canada), okadaic acid ammonium salt was purchased from LC Laboratories (Woburn, MA, U.S.A.), and sodium orthophosphate from BDH Chemicals (Darmstadt, Germany). The reagents that were not soluble in water (okadaic acid, H-7, W-7, and staurosporine) were dissolved in a small volume of ethanol (1 mg ml^{-1}, 6.5 mg ml^{-1}, 25 mg ml^{-1}, and 1mg ml^{-1}, respectively) before preparing an aqueous solution. The same volume of ethanol was added to the corresponding control solution.

2.2.3. Steady-state root water flow (Q_v) measurements

The steady-state root water flow (Q_v) was determined using the hydrostatic pressure method as described by Wan and Zwiazek (1999). Roots were excised about 1 cm above the root collar and immersed in distilled water in 250 ml polyvinyl chloride containers. The containers were placed inside the pressure chambers (PMS Instruments, Corvallis, OR, U.S.A.) with the cut end of the root protruding through a rubber stopper fitted in the lid. The chambers were pressurized to 0.3 MPa and this pressure was maintained constant during the measurements. A graduated pipette (1 ml) was attached through a small piece of rubber tubing (1-2 mm diameter) to the cut end of the root and water coming out of the root was measured at five minute intervals. The mean volume of water expressed over time represented the rate of water flow (Q_v). The initial water flow was measured for 30 min and then, the pressure was released and distilled water was replaced with different concentrations of chemical inhibitors or fresh distilled water for control roots. The roots were pressurized again to 0.3 MPa and the Q_v was measured for another 60 to 90 min. The mean flow rate for the first 30 min was used to normalize the data for each root system. The mean Q_v over the first 30 min varied from 3 to 17 x 10^{-13} m^3 s^{-1}.

For OA and W-7 measurements, the Q_v was measured for 1 h after the inhibitors were added. Subsequently, the pressure was released and the roots were incubated for 4 h in OA and 2 h in W-7 aerated solutions or water (control). The flow rates were measured again for 1 h with roots placed in distilled water. In a different experiment, Q_v was measured in roots treated with OA and distilled water and then the root bathing solutions were changed to 5 mM KF and Q_v was recorded again for another 60 min.

2.2.4. Root respiration measurements

Root respiration was measured in selected treatments (5 mM 6-DMAP, 5 mM KF, 5 mM Na_3VO_4, 1 μM OA, and for the corresponding controls) as oxygen uptake using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.). Roots were removed from the solution culture and enclosed in an airtight cylinder containing 340 ml of previously aerated distilled water. The initial oxygen level was approximately 7-8 mg l^{-1}. The solution was kept continuously stirred with a magnetic stirrer. Oxygen uptake was measured every 5 min for 25-30 min. Root respiration rate was calculated from the slope of oxygen uptake versus time. Respiration rates were recorded before and after the inhibitor treatments (applied similarly with Qv measurements) to normalize the data for each root system. Six to nine root systems were used for each treatment.

2.2.5. Acid phosphatase assay

An acid phosphatase assay was carried out as described by Zwiazek and Shay (1988), for KF, Na_3VO_4 and OA *in vivo* or *in vitro* using a different set of plants. Briefly, 1g of fresh root tissue was harvested and ground in a mortar with sand and 15 ml cold 50 mM Tris-HCl (pH 7.4), 1% (w/v) polyvinvyl pyrolidone (PVP-40), and 1 mM dithiothreitol (DTT). The homogenate was filtered through 6 layers of cheesecloth and used immediately. All extraction steps were carried out on ice.

Acid phosphatase activity was measured using p-nitrophenyl phosphate (P-NPP) as a substrate. The standard assay solution contained 0.6 mM P-NPP in 50 mM acetate

buffer (pH 5.0). The effect of okadaic acid on phosphatase activity was also assayed using a modified assay solution containing 0.6 mM P-NPP in 50 mM Tris-HCl (pH 7.4). Serine/threonine phsohatases inhibited by OA are assayed with different buffers having a pH of 6.8 of 7.4 (Serine/threonine phosphatase assay system, Promega, Madison, WI, U.S.A.). For the acid phosphatase activity assay in Na_3VO_4-treated extracts, the pH of the solution was adjusted to 5.0 with acetic acid.

2.2.5.1. *In vitro* acid phosphatase assay

For *in vitro* phosphatase assay, 50 µl of the control plant root extract was added to 2 ml assay solution supplied with 50 µl of KF or Na_3VO_4 to give a final concentration of 0.5, 1, and 5 mM KF or Na_3VO_4 in the reaction mixture. Similarly, 50 µl control root extract was added to 2 ml assay solution supplied with 50 µl OA solution to give a final concentration of 0.01 µM, 0.1 µM, or 1 µM OA in the reaction mixture. Distilled water (50 µl) was added to the control test tubes. The blank test tubes contained 50 µl of the homogenization medium instead of the root extract. The reaction mixtures were incubated in a water bath at 30°C for 25 min and the reaction was stopped with 2 ml 10% (w/v) Na_2CO_3. For each chemical inhibitor used, roots from six different plants were harvested (n = 6). The absorbance of the reaction mixtures was measured at 410 nm against the corresponding blank using a spectrophotometer (Ultrospec III, Pharmacea LKB Biotechnology, Uppsala, Sweden). Values obtained for the control tubes were used to normalize readings for the corresponding treatment test tubes.

2.2.5.2. *In vivo* phosphatase assay

The *in vivo* acid phosphatase assay was carried out for the roots treated with 1 μM OA for the Q_v measurements. After Q_v measurements, the roots were immediately used for the acid phosphatase assay as described above. Root extract (50 μl) was added to 2 ml of the standard assay solution and then the test tube was incubated for 25 min and acid phosphatase activity measured as described above. The activity was expressed as change in absorbance per minute.

2.2.6. Statistical analysis

For the steady-state root water flow and for okadaic acid respiration measurements, statistical analyses were performed using the Mixed Procedure of SAS (version 8.1, SAS Institute, Cary, NC). Steady-state root water flow rates, and root respiration characteristics were analyzed as a repeated measures analysis of variance. The fixed effects in the model were the treatments (different chemical inhibitors applied in the root bathing solution). For completion, experiments had to be carried out in consecutive days. The root water flow measurements were made at times 5, 10,…., 60; 5, 10, 15, …, 90; 5, 10, …, 60, 305, 310, …,360; or 5, 10, …60, 185, 190,…, 240 min after the treatments were applied. In all statistical models, a Kenward-Roger option was used to calculate denominator degrees of freedom. The Variance-Covariance Matrix chosen for each model was an iterative process and based on the Schwarz's Baysian Criterion (SAS 2000). Least Square Means and standard errors were calculated for treatments, days, time

and treatment*time interaction effects and the Least Square Means separated using a pdiff option. The statistical model was:

$$Y_{ijkl} = \mu + T_i + D_j + P_k + TP_{ik} + e_{ijkl}$$

Where Y_{ijkl} = value of individual observation

μ = overall mean

T_i = effect of i^{th} treatment (i = the level of treatments)

D_j = effect of j^{th} day (j = the level of day)

P_k = effect of k^{th} period (k = the level of period 5, 10,….., 60 min; 5, 10, ….., 90 min; 5, 10, …, 60, 305, 310, …,360; or 5, 10, …60, 185, 190,…, 240 min)

TP_{ik} = interaction effect of i^{th} treatment with k^{th} period

e_{ijkl} = random error (l = 1, 2,….., number of replicates)

(T*D interaction was not introduced due to the lak of degrees of freedom)

Root respiration data for other inhibitors and phosphatase assay data were analyzed using GLM Procedure of SAS (version 8.1, SAS institute, Cary, NC, USA). The statistical model was:

$$Y_{ijk} = \mu + T_i + D_j + TD_{ij} + e_{ijk}$$

Where Y_{ijkl} = value of individual observation

μ = overall mean

T_i = effect of i^{th} treatment (i = the level of treatments)

B_j = effect of j^{th} day or group (j = the level of day or group)

TB_{ij} = interaction effect of i^{th} treatment with j^{th} day or group

e_{ijk} = random error (l = 1, 2,….., number of replicates)

Least Square Means and standard errors were calculated for treatments, days or

group, and treatment*day interaction effects and the Least Square Means separated using a pdiff option. All differences were tested at the $P \leq 0.05$ level.

The number of seedlings measured in one day for one treatment varied from 1 to 5 depending on the treatment.

2.3. Results

2.3.1. Root morphology

When harvested for measurements, roots of the solution culture-grown seedlings had mostly white and pink-colored roots with relatively few branches and woody roots.

2.3.2. Steady-state root water flow (Q_v) and root respiration

2.3.2.1. Phosphorylation inhibitors

H-7. The steady-state root water flow was measured for 60 min after adding 50 μM H-7 to the root incubation solution. When compared to the control, H-7 treatment had no significant effect on the Q_v (Figure 2.1-A).

Staurosporine. The rate of root water flow (Q_v) was not affected by 0.7 μM staurosporine compared with control roots (Figure 2.1- B).

6-DMAP. The two concentrations of 6-DMAP used, 1 mM and 5 mM, had different effects on Q_v (Figure 2.2-A). The lower, 1 mM 6-DMAP concentration resulted

in a small increase in the Q_v values and the difference became significant after 30 min from the treatment initiation ($P = 0.0216$). After 35 min, the differences between the rates of water flow between the control and 1 mM 6-DMAP treatments were no longer significant.

A higher, 5mM, concentration of 6-DMAP decreased the Q_v values, and the difference became significant after 55 min as compared to control roots following treatment application ($P = 0.0143$). The Q_v values continued to be significantly lower from the control throughout the treatment and declined by 26% compared with the control level after 90 min of treatment ($P < 0.0001$) (Figure 2.2-A).

Following treatment application, root respiration rates decreased by 38% in control roots and 34% in roots treated with 5 mM 6-DMAP (Figure 2.2-B). However, the difference between the two was not significant ($P = 0.3521$).

2.3.2.2. Dephosphorylation inhibitors

Potassium fluoride (KF). Both 1 mM and 5 mM KF resulted in a decrease of Q_v over time (Figure 2.3). After 5 min exposure, the application of 1 mM KF resulted in an 8% decrease ($P = 0.0162$) of Q_v compared to the control. The Q_v values continued to decrease steadily and were significantly lower than controls for all treatment durations. The higher, 5 mM KF, concentration induced a 13% decrease within 5 min ($P < 0.0001$), and Q_v continued decreasing over time (Figure 2.3).

Root respiration decreased over time with 43% in control and 45% in 5 mM KF-treated roots. However, the difference between KF-treated and control roots was not

statistically significant (P = 0.7526) (Figure 2.4-B).

Sodium orthovanadate (Na_3VO_4). During the first 15 min of 1 mM Na_3VO_4 treatment, the decrease in Q_v was not statistically significant from the control (P = 0.2971 and P = 0.1305 after 5 and 15 min, respectively). The decrease became significant after 20 min (P = 0.0359), and reached 23% after 60 min (P < 0.0001). A higher, 5 mM Na_3VO_4, concentration resulted in an immediate and significant decrease in Q_v rates when compared to the control (P < 0.0001). The decline was 19% within 5 min and 43% after 60 min (P < 0.0001) (Figure 2.3).

Root respiration rates were measured for roots treated with 5 mM Na_3VO_4 (Figure 2.4-A). Both control and Na_3VO_4-treated roots showed a decrease in root respiration, following pressurization. Compared to the initial values, the decrease was 28% for control and 21% for Na_3VO_4-stressed roots. There was no significant difference between control and Na_3VO_4 treatments (P = 0.2546).

Sodium phosphate (Na_3PO_4 x 12 H_2O). A solution of 5 mM Na_3PO_4 x 12 H_2O was used as a Na control for the 5 mM Na_3VO_4 treatment (Figure 2.3). This solution resulted in a 21% decline in Q_v within 5 min and 35% decline after 60 min, when compared with distilled water (P < 0.0001). The rates of root water flow for Na_3VO_4-treated roots were only slightly lower than those for Na_3PO_4 x 12 H_2O-treated roots. However, Q_v became significantly lower in 5 mM Na_3VO_4 compared with 5 mM Na_3PO_4 x 12 H_2O treatment: after 35 min 6%, (P = 0.0459), and 50 min 8%, (P=0.0055).

Okadaic acid (OA). Overall, 0.2 μM okadaic acid had no significant influence on root water flow properties, even after incubating the roots in the OA solution for 4 hours

(Figure 2.5-A). A higher concentration of OA (1 μM) decreased Q_v after 80 min reaching 20-31% significant decrease after 4 h (Figure 2.6-A).

A different OA experiment involved the application of 5 mM KF to both control and OA-treated roots, after 60 min of OA treatment (Figure 2.5-B). The application of 5 mM KF to both control and OA-treated roots had no significant effect on Q_v. However, following KF treatment, OA-treated roots maintained up to 8% higher Q_v values compared with control roots, but this difference was not significant.

Roots treated with 1 μM OA maintained significantly higher respiration rates compared with control roots (Figure 2.6-B). After 1 h treatment, control roots showed a 32% decrease, while the OA-treated roots showed a 12% decrease in respiration rates ($P = 0.0124$). Following 4 h incubation and 1 h pressurization, the root respiration rates decreased by a total of 43% for control and 23% for 1 μM OA treatment ($P = 0.0105$).

2.3.2.3. Calmodulin inhibitor (W-7)

Both 50 and 150 μM W-7 decreased root Q_v compared with control by up to 10% up to 90 min, but the difference was not significant at $P \leq 0.05$ level (Figure 2.7). Also, after 1-h treatment under pressure followed by 2-h incubation in treatment solutions, there were no significant differences between Q_v of W-7-treated and control roots (Figure 2.7).

2.3.3. Acid phosphatase assay

Potassium fluoride had a profound impact on the *in vitro* activity of acid phosphatase in root extracts (Figure 2.8-A). When compared with control, the activity of acid phosphatase decreased by 42% in 0.5 mM KF, 54% in 1 mM KF and 69% in 5 mM KF treatments ($P < 0.0001$).

Sodium orthovanadate significantly ($P < 0.0001$) decreased the *in vitro* activity of acid phosphatase in root extracts (Figure 2.8-B). When the pH of the vanadate solution was not adjusted, 0.5 mM, 1 mM, and 5 mM Na_3VO_4 resulted respectively in 60%, 61%, and 45% decrease in the acid phosphatase activity. Since the addition of Na_3VO_4 solution changed the pH of the assay medium, another assay was done with the pH of the Na_3VO_4 solution lowered to the pH of the assay medium (pH 5.0). There was also a significant ($P < 0.0001$) decrease in the acid phosphatase activity with increasing Na_3VO_4 concentration (47%, 55% and 58%, respectively, for 0.5 mM, 1 mM, and 5 mM Na_3VO_4).

Okadaic acid's effect on acid phosphatase activity was also assayed *in vitro* (Figure 2.9-A). At pH 5.0 of the reaction mixture, the tested concentrations of OA had no effect on acid phosphatase activity. When the pH was increased to 7.4, phosphatase activity barely decreased with increasing OA concentrations. However, this decrease was not significant at $P \leq 0.05$ level when compared with control.

A small decrease in the acid phosphatase activity was also observed in the extracts from plants treated with 1 μM OA, although the decrease was not statistically significant ($P = 0.2076$) (Figure 2.9-B).

2.4. Discussion

Phosphorylation inhibitor 6-DMAP and dephosphorylation inhibitors KF, Na_3VO_4, and OA reduced Q_v but not root oxygen uptake. Therefore it appears that phosphorylation and dephosphorylation events contributed to the decreased Q_v observed in the present study but not root respiration. Other inhibitors used (H-7 and staurosporine) had no effect on Q_v, suggesting that the concentrations used may not be effective on root water uptake.

2.4.1. Effects of protein phosphorylation inhibitors on steady-state root water flow (Q_v) and root respiration

2.4.1.1. Effects of 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7)

The concentration of H-7 (50 μM) used in the present study did not affect Q_v. This reagent is an inhibitor of protein kinase C, cyclic nucleotide dependent protein kinase, and Ca^{2+}/calmodulin dependent protein kinases (Hidaka et al. 1984, Park and Chae 1990). In spinach leaves, phosphorylation of PM28A occurred on Ser-274, seven amino acids from the C terminus of the protein, within a consensus phosphorylation site (Ser-X-Arg) for vertebrate protein kinase C (Johansson et al. 1996).

The concentration of H-7 used in the present study was within the range of concentrations used in other *in vivo* and *in vitro* studies to inhibit protein phosphorylation. The concentrations ranged from 0.001 to 1 μM for purified enzymes (Chandok and Sopory 1996, Chandok and Sopory 1998), 1 to 100 μM for *in vitro* assay

of kinase activities (Yang and Komatsu 2000), and 10 to 300 μM when protoplasts, tissues or plant organs were used (Lee and Assman 1991, Leitner-Dagan and Weiss 1999, Deshpande et al. 1997).

The plants used in the present study were grown under standard, non-stress conditions. Therefore, their tissues could have contained high amounts of ATP. Since H-7 competes with ATP, higher concentrations of H-7 are required to inhibit protein kinases when cells or tissues contain high concentrations of ATP (Hidaka et al. 1984). The penetration of H-7 inside cells is also dependent on its concentration (Hidaka et al. 1984). Consequently, it is possible that the concentration used in the present study was not adequate to have an effect on root water uptake under the study conditions.

2.4.1.2. Staurosporine

Staurosporine (0.7 μM) had no effect on root water flow properties in the present study. Staurosporine is an antifungal alkaloid that is known to inhibit protein kinase C, cAMP-dependent protein kinase (PKA), and protein tyrosine kinase (PTK) (Tamaoki et al. 1986, Yoshida and Anraku 2000, Tamaoki 1991). In previous studies, staurosporine was used in concentrations ranging from 0.17 to 200 μM for plant organs (Baskin and Wilson 1997, Rakwal et al. 2001, Agrawal et al. 2002, Li and Komatsu 2000), 2 to 5 μM for cell culture (Chen et al. 2001), and 1 to 1.5 μM for kinase assays (Cazalé et al. 1999, Mori et al. 2000). A staurosporine derivate, staurosporine aglycol, decreased the water uptake of PM28A when expressed in *Xenopus* oocytes (Johansson et al. 1998). Since

staurosporine is an inhibitor with a broad-spectrum activity (Vitrac et al. 2000) it is possible that different concentrations are required to induce a response in aspen roots.

2.4.1.3. 6-dimethyl aminopurine (6-DMAP)

Two concentrations of 6-DMAP were used. While 1 mM 6-DMAP increased Q_v, 5 mM 6-DMAP decreased Q_v. The root oxygen uptake was not affected by 5 mM 6-DMAP. 6-dimethyl aminopurine is derivative of puromycin and is a broad-spectrum protein kinase inhibitor (Rebhun et al. 1973, Szöllösi et al. 1993). The concentrations of 6-DMAP used in the previous studies ranged from 0.5 μM to 5 mM for cell culture (Rugini and Caricato 1995, Mizuno 1992), tissues and plant organs (Mizuno 1994, Mathooko et al. 1998). In tobacco cells, 6-DMAP inhibited protein phosphorylation at the plasma membrane level (Mathieu et al. 1996). When phosphorylated by an unknown and probably membrane-bound protein kinase, some aquaporins display higher water permeability (Johansson et al. 1998).

In the present study, 6-DMAP acidified the root medium from pH 6.0 in control to pH 5.5 for 1 mM and pH 5.0 for 5 mM 6-DMAP. Low pH has been demonstrated to decrease the rates of water flow through the apoplast (Meychik and Yermakov 2001). However, in the present study, 1 mM 6-DMAP increased the water uptake by 10%. Although the physiological significance of this increase is probably small, it is possible that the stimulation of water uptake was due to the activation of aquaporins by low pH, which has been observed for some mammalian aquaporins (Németh-Cahalan and Hall 2000, Yasui et al. 1999). Respiration may affect the function of aquaporins through

phosphorylation (Kamaluddin and Zwiazek 2001, Kamaluddin and Zwiazek 2002). However, the higher, 5 mM 6-DMAP concentration used in the present study resulted in a decrease in Q_v by 26% after 90 min of treatment, while root respiration rates remained at the control level. 6-DMAP has been used to inhibit different animal and plant protein kinases (Neant and Guerrier 1988, Mathieu et al. 1996). Therefore, it is possible that the effect of 6-DMAP on Q_v in the present study, was mediated through inhibitory effects on the protein kinases that phosphorylate the aquaporins.

2.4.2. Effects of protein dephosphorylation inhibitors on the steady-state root water flow and root respiration

2.4.2.1. Potassium fluoride

Potassium fluoride induced a rapid and substantial concentration-dependent decrease in the root water flow of aspen seedlings. In the present study, 5 mM KF resulted in a 37% decrease in Q_v within 60 min. The rapid (observed within 5 min) and increasing over time inhibition of Q_v by KF suggests that the response likely involved root aquaporins. Kamaluddin and Zwiazek (2003) suggested that the decrease in the steady-state root water flow induced by 5 mM KF was mediated through a decrease in respiration, which probably affected the activity of water channel proteins. However, in the present experiment, the decrease in water uptake of fluoride-treated roots was not accompanied by a decrease in root O_2 uptake. Therefore, decreased water transport in roots was likely due to processes other than root respiration.

Fluoride has been shown to alter the lipid composition of plasma membrane (Rakowski 1997). Since the stability and function of proteins are influenced by the composition of membranes, it is plausible that the effect on membrane lipids could be responsible for the observed decrease in Q_v. However, there has been little research done on lipid-aquaporin interaction in cell membranes.

Fluoride can inhibit the activity of phosphoseryl and phosphothreonyl protein phosphatases and the activity of acid phosphatases (Zwiazek and Shay 1988, Struglics et al. 2000). In the present experiment, KF had a great impact on the *in vitro* acid phosphatase activity, suggesting that the concentrations used for the whole root system could act through the same inhibitory mechanism on different protein phosphatases. The mode of fluoride action on plants is not well understood. Some possible mechanisms could include direct or indirect inhibition of H^+-ATPase (Rakowski et al. 1995) resulting in a decrease in cell membrane permeability due to the H^+ blocking of aquaporins (Gerbeau et al. 2002). It has been shown that fluoride can stimulate the activity of G proteins that are mediators in transmembrane signaling (Palme 1996, Graham et al. 1999). Therefore, fluoride could also interact with an unidentified receptor (mediator in transmembrane signaling) that triggers a decrease in water permeability in response to different stresses (Gerbeau et al. 2002). By affecting a number of protein phosphatases, fluoride could also have an effect on the synthesis of aquaporins or their insertion into the membranes.

At the plant tissue level, fluoride can induce calcium deficiency (Ballantyne and Glover 1981), most likely by precipitating Ca^{2+} (Brautigan and Shriner 1988). Although calcium has a protective effect on the plasma membrane (Carvajal et al. 2000), Gerbeau

et al. (2002) showed that Ca^{2+} could directly block aquaporins. Fluoride ions also increased the plasma membrane permeability in *Arabidopsis* suspension cells, probably by reducing the intracellular free Ca^{2+} concentration by co-precipitation (Gerbeau et al. 2002). Some aquaporins are phosphorylated by a Ca^{2+}-dependent protein kinase and, when phosphorylated, aquaporins display higher water transport activity (Johanson et al. 1996). Subsequently, in the present study, precipitation of Ca^{2+} could potentially affect protein kinases that phosphorylate the aquaporins.

2.4.2.2. Sodium orthovanadate

Sodium orthovanadate brought about an immediate and large inhibition of Q_v, with no effect on root respiration. Although a decrease in root respiration was accompanied by reduced water transport in roots, presumably through aquaporins (Zhang and Tyreman 1991, Kamaluddin and Zwiazek 2001, Kamaluddin and Zwiazek 2003), in the present study Na_3VO_4 did not affect root O_2 uptake. In *Allium cepa*, 1 mM Na_3VO_4 reversibly inhibited root growth after 2 hours of exposure (Hidalgo et al. 1988). In the submerged angiosperm *Sparganium emersum*, 1 mM vanadate reduced the long distance water transport (Pedersen 1993) that is dependent on energy conversion in roots. It is plausible that water channel proteins were responsible for the inhibition of Q_v by Na_3VO_4 that was observed in the present study.

Sodium orthovanadate changed the pH of the solution to about 10.5 for both concentrations used, yet for 1 mM Na_3VO_4, the effect on root water flow properties was significant only after 20 min while for 5 mM Na_3VO_4 the effect was immediate. The

swelling coefficient of the cell walls increases with increasing pH which, in turn, can increase hydraulic conductance of the cell walls or increase the apoplastic volume flow of water through roots (Meychic and Yermakov 2001). However, in the present study, root water flow was inhibited suggesting that the effect was likely on the cell-to-cell transport and not on the apoplastic transport. A high pH could also inactivate the enzymes associated with the plasma membrane such as ATPases (Widell and Larson 1990). In addition to the negative effects on ion uptake, a high pH in the root environment has been shown to affect the functioning of the root cell membranes, probably by increasing the permeability of the plasma membrane (Zieslin and Abolitz 1994), analogous to the effect of a low pH (Yan et al. 1992).

In the present study, the *in vitro* effects of Na_3VO_4 on acid phosphatase activity were also assayed. Regardless of the pH of the reaction medium, Na_3VO_4 inhibited the activity of acid phosphatases. While a high pH could also inhibit most of the phosphatases, the inhibition obtained at low pH is likely due to the inhibitory effect of vanadate on acid phosphatases in plant cells (Hidalgo et al. 1988). Therefore, at the whole organ level, Na_3VO_4 could act through the same inhibitory mechanisms on protein phosphatases. Orthovanadate also inhibits the activity of protein tyrosine phosphatases (PTPases) (Brautigan and Shriner 1988). The plant PTPases may function in the upstream MAPK protein kinase pathway in response to a number of extracellular signals including salinity and low temperature (Luan 2000). However, the receptors that sense these environmental stresses and their links with water transport through aquaporins are not known.

Sodium othovanadate solution contains a relatively high amount of sodium. Sodium

may compete with Ca^{2+} for membrane binding sites, which in turn can alter root hydraulic conductivity (Martinez-Ballesta et al. 2000). Therefore, the inhibition of water-channel activity in NaCl-treated plants or protoplasts, can also be due to a direct action of salt on the proteins that regulate aquaporin opening and closing (Martinez-Ballesta et al. 2000). In the present study, both 5 mM Na_3VO_4 and 5 mM $Na_3PO_4 \times 12H_2O$ treatments resulted in approximately the same extent of Q_v inhibition. There were significant differences between the two treatments for the last three measurement times, which could suggest different mechanisms of action for the two inhibitors. Nonetheless, inorganic phosphate is also a common competitive inhibitor of protein phosphatases (Kutuzov and Andreeva 2001).

Vanadate is a well-known inhibitor of the plasma membrane P-type ATPases (H^+, Ca^{2+}, Na^+/K^+, H^+/K^+) (Widell and Larsson 1990, Michelet and Boutry, 1996). Consequently, the inhibitory effect on root water flow could be due to the inactivation of the plasma membrane ATPases. So far, there is no evidence of a direct connection between ATPases and water channel proteins either in the tonolast or plasma membrane. As suggested by Kjellbom et al. (1999), water channels of the plasma membrane and tonoplast may play important roles in water flux of the cell and in buffering of osmotic fluctuations of the cytosol, respectively. In *Mimosa pudica*, an increase in the expression of vacuolar aquaporin and H^+-ATPase accompanied the differentiation of motor cells and motile function of pulvini (Fleurat-Lessard et al. 1997). Another study (Shiratake et al. 2001) showed that during grape berry development, there was a similarity between the fluctuations of the amount of P-ATPase and plasma membrane aquaporins (PAQ), and the same trend was seen for their counterparts in the tonoplast. According to Baiges et al.

(2002), an activated Ca^{2+}-ATPase that would keep calcium outside the cytoplasm would reduce the phosphorylation and the activity of PM28A aquaporin. Therefore, it is conceivable that there is a link between aquaporins and plasma membrane ATPases. So far, the studies showed that both proteins were required for the same process at the cellular level. However, this does not exclude a direct link among the functions of both proteins. Water transport in roots is coupled with the uptake of nutrients (Clarkson et al. 2000). The nutrient transport across membranes is energy-dependent and coupled with the activity of plasma membrane H^+-ATPase (Sakano et al. 1992). As shown by Tazawa et al. (2001), inhibitors of K^+ channels also had an inhibitory effect on water flow across membranes. Cells at full turgor or with intense transport activity, such as guard cells, stellar cells, and transfer cells, exhibit high activity of the plasma membrane H^+-ATPase that extrudes H^+ to generate the electrochemical gradient required for the uptake of solutes (Morsomme and Boutry 2000). As suggested by Gerbeau et al. (2002), this may be associated with local cytosolic alkalization along the plasma membrane (Gout et al. 1992), which in turn may increase cell L_p and favour quasi-isoosmotic water flow linked to solute transport. On the other hand, hypoxic stress has been shown to acidify the cytoplasm (Gerendás and Ratcliffe 2002), and also to reduce root water uptake (Maggio and Joly 1995, Zhang and Tyreman 1991), probably by a direct blockage of water-channel by H^+ ions (Gerbeau et al. 2002). Therefore, in the present study, it is conceivable that an inhibition of plasma membrane H^+-ATPase could result in acidification of the cytoplasm and inhibition of the activity of water channel proteins.

A problem with the *in vivo* studies is that vanadate may penetrate slowly into intact cells (Ball et al. 1987). However, in the present study, the rapid response of Q_v to

Na_3VO_4 was likely due to a direct effect of Na_3VO_4 on root cells. Nevertheless, it is possible that the combined effects of the above factors contributed to some extent to the observed inhibition of Q_v.

2.4.2.3. Okadaic acid

In the present study, 0.2 μM and 1 μM OA had no immediate effect on root water flow properties. The higher, 1 μM OA concentration, decreased Q_v only after 4 h while root O_2 uptake was increased. Since okadaic acid is hydrophobic, it can rapidly enter cells and increase the phosphorylation status of many proteins within minutes (Cohen et al. 1990). In intact cells, at least 1 μM OA was required to totally block protein phosphatases 1 and 2A (Hardie et al. 1991), which are abundant in plant cell extracts (MacKintosh and Cohen 1989, Hardie et al. 1991). In renal collecting duct cells, OA has been shown to induce AQP2, translocation to the apical membrane, independently from AQP2 phosphorylation (Valenti et al. 2000). In plants, 5 μM OA increased the water permeability of oocytes expressing the spinach leaf PM28A aquaporins (Johansson et al. 1998). However, as suggested by Hardie et al. (1991), OA can only be effective if the kinase whose effect it reverses is not fully active. Therefore, in healthy unstressed plants, such as the seedlings used in the present study, the activity of protein kinases could be high and OA might not be very effective.

When cells are incubated with OA for a short time, no toxic effects are observed (Cohen et al. 1990). However, in long-term incubation, toxic effects can appear due to an increase in protein phosphorylation (Hardie et al. 1991). Therefore, the decrease in Q_v

obtained after 4 hours with 1 μM OA was probably a secondary effect. However, this effect was not likely mediated through respiration, since O_2 uptake was greater than in OA-treated compared with control roots. OA-treated roots maintained higher respiration rates even after the inhibitor was removed and roots were pressurized for another hour in distilled water.

Similarly to the present study, OA had no effect on the water permeability of isolated protoplasts from melon plants (Martinez-Ballesta et al. 2000). However, when 5 μM OA was added to the NaCl-treated protoplasts, the protoplasts maintained higher osmotic permeability than those treated with NaCl alone (Martinez-Ballesta et al. 2000). In the present study, roots were treated with KF after they had been treated with 0.2 μM okadaic acid. However, there was little difference in the Q_v values compared with the control that was treated only with KF. It is likely that a higher concentration of okadaic acid is required to have an immediate and significant effect on aquaporin dephosphorylation and root water flow. It is also possible that KF affected cells in a different manner as NaCl in the melon protoplasts (Martinez-Ballesta et al. 2000).

In the present study, the effect of OA on the activity of phosphatases was assayed at two different pH's, with no effect on the phosphatase activity. Similarly, the roots treated with OA showed no effect on acid phosphatase activity. It has been reported that OA has no effect on the activity of acid and alkaline phosphatases (Hardie et al. 1991). However, in the present study, OA likely affected other processes than acid phosphatases since Q_v declined after 4 h.

2.4.3. Effects of calmodulin inhibitor (W-7) on root water flow properties.

In the present study, a calmodulin inhibitor had no significant effect on the root water flow. A small Q_v decrease observed in W-7-treated roots was not statistically significant. In plants, the calmodulin inhibitor W-7 has been used in a variety of concentrations ranging from 0.5- 500 μM for the *in vitro* studies (Estruch et al. 1994, Romeis et al. 2000, Deswal et al. 2000), and 50-100 μM for plant organs or segments (Yang and Komatsu 2000, Leitner-Dagan and Weiss 1999). Although calmodulin is involved in a variety of cellular responses activating numerous enzymes in plants (Roberts and Harmon 1992), little is known about its involvement in aquaporin regulation.

Much of the evidence regarding the involvement of calmodulin in regulating water permeability of the plasma membrane comes from the mammalian systems. In oocytes, three calmodulin inhibitors, including W-7, increased the permeability of AQP0 (Németh-Cahlan and Hall 2000), suggesting that Ca^{2+} may act through calmodulin. However, the study of Chou et al. (2000) suggests that vasopressin-mediated AQP2 trafficking to the apical plasma membrane takes place via calmodulin-dependent mechanisms. In the nematode-trapping fungus *Arthrobotrys dactyloides*, activated G proteins lead to an increase in cytoplasmic Ca^{2+}, activation of calmodulin and, finally, to the opening of water channel proteins (Chen et al. 2001). However, in *Brasica rapa* plants, 0.2 μM W-7 did not have any effect on root hydraulic conductivity decrease that was recorded at low temperature (Bigot and Boucaud 2000).

Experiments using a higher concentration of W-7 in intact tissues may be needed to

clarify the effect of this inhibitor on the water uptake by roots. On the other hand, the effect of higher concentration of W-7 on calmodulin may be questionable since W-7 can interact in a calcium-dependent manner with other calcium-dependent proteins (Roberts et al. 1986). Consequently, at higher concentrations W-7 may not be able to distinguish between calcium-calmodulin stimulated process and other processes (Roberts et al. 1986). Plants CDPK can also be inhibited by W-7 (Sopory and Munshi 1998), and CDPK and Ca^{2+} have been suggested to regulate aquaporin activity (Johansson et al. 1998).

2.5. Conclusions

In conclusion, the present study suggests the involvement of phosphorylation and dephosphorylation events in modulating the root water uptake. Most of the chemical inhibitors used resulted in a decrease in root water flow while root respiration was not affected. However, it is not clear whether phosphorylation/dephosphorylation affected the function of aquaporins, their synthesis, or whether phosphorylation stimulated membrane insertion of aquaporins through a mechanism similar to that in animal cells. The mammalian AQP2 translocation to the plasma membrane is conveyed through cytoskeletal rearrangements (van Balkom et al. 2002). In plant roots, inhibitors of protein kinases and phosphatase can disorganize cortical microtubules (Baskin and Wilson 1997) and stimulate radial expansion of cells. It is also possible that decreased Q_v was a secondary response due to the inhibition of ATPases or other protein receptors in the root plasma membranes. Since the effects of different chemical inhibitors on the apoplast and on the permeability of the lipid bilayer have not been studied, future work should

consider these in addition to water transport. As pointed out by Meychik and Yermatov (2001), swelling of the root cell walls induced by diferent external or internal conditions is one of the mechanisms regulating water flow in plant roots. Phosphorylation and dephosphorylation processes can also affect the cellular traffic via plasmodesmata (Blackmann and Overall 2001), although at external pressures higher than 0.2 MPa plasmodesmata have been shown to close (Oparka and Prior 1992).

Since the air spaces will fill with water in roots that are placed under pressure, the lack of air-filled spaces has raised the question that roots may suffer from hypoxic conditions that could affect their transport characteristics (Markhart and Smit 1990). In the present study, this could explain the decrease in root respiration for both control and treated plants. However, in control roots, a decrease in respiration was not accompanied by a decrease in Q_v.

In the present study, different inhibitors were used to test their effects on root water flow properties. However, steady-state root water flow is a macro parameter that probably involves the participation of different cellular processes. Therefore, the results of this study should be interpreted with caution and doubled with studies at the cellular level.

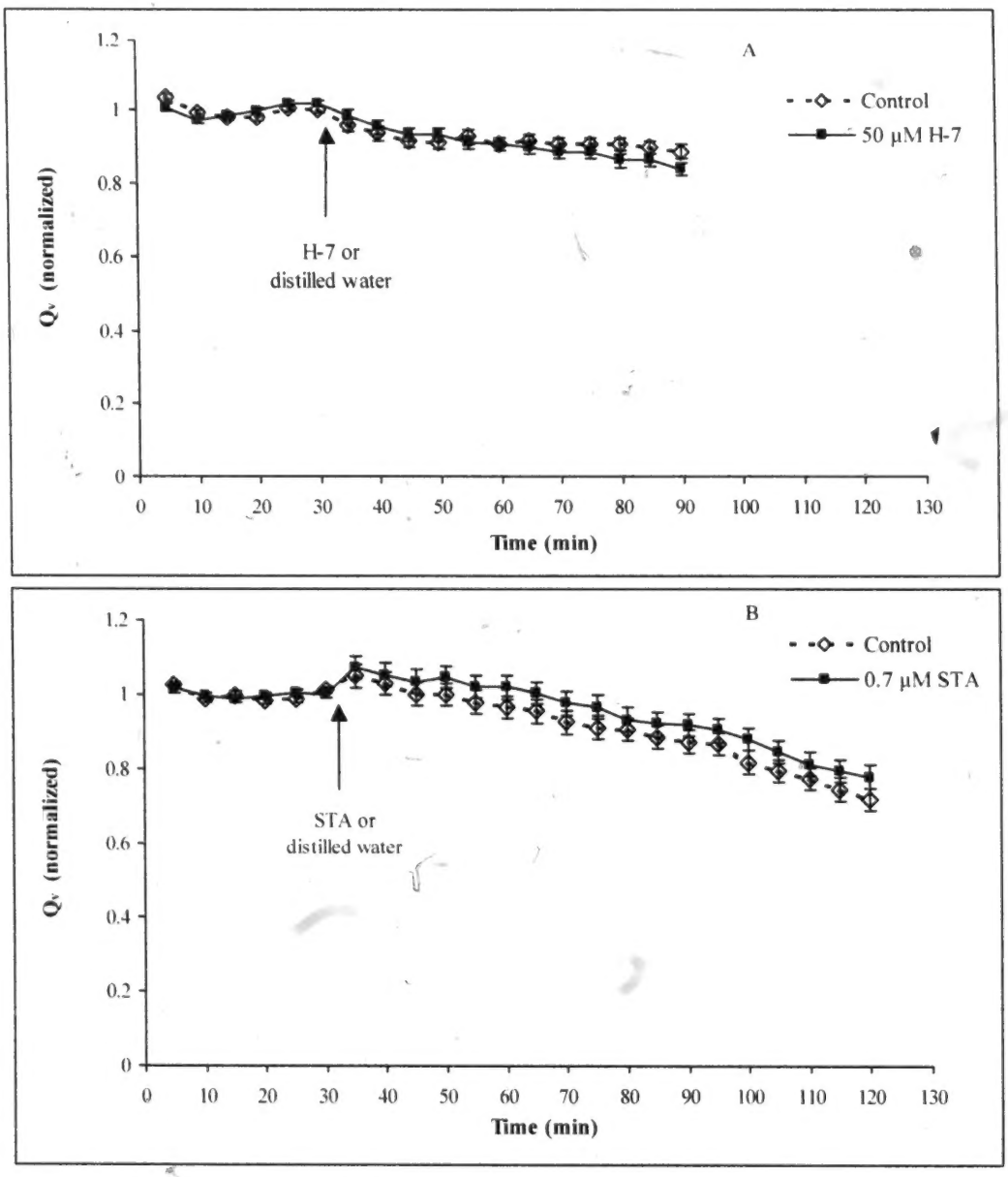


Figure 2.1 Time course of root water flow (Q_v) in response to phosphorylation inhibitors 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) (A) and staurosporine (STA) (B). Q_v was normalized with the mean rate over the initial 30 min before chemical inhibitors were applied. Data points represent least square means (n=6) ± SE. Arrows indicate the time of application of different chemical inhibitors. The differences between corresponding control and inhibitor treatment were not significant at P≤0.05 level.

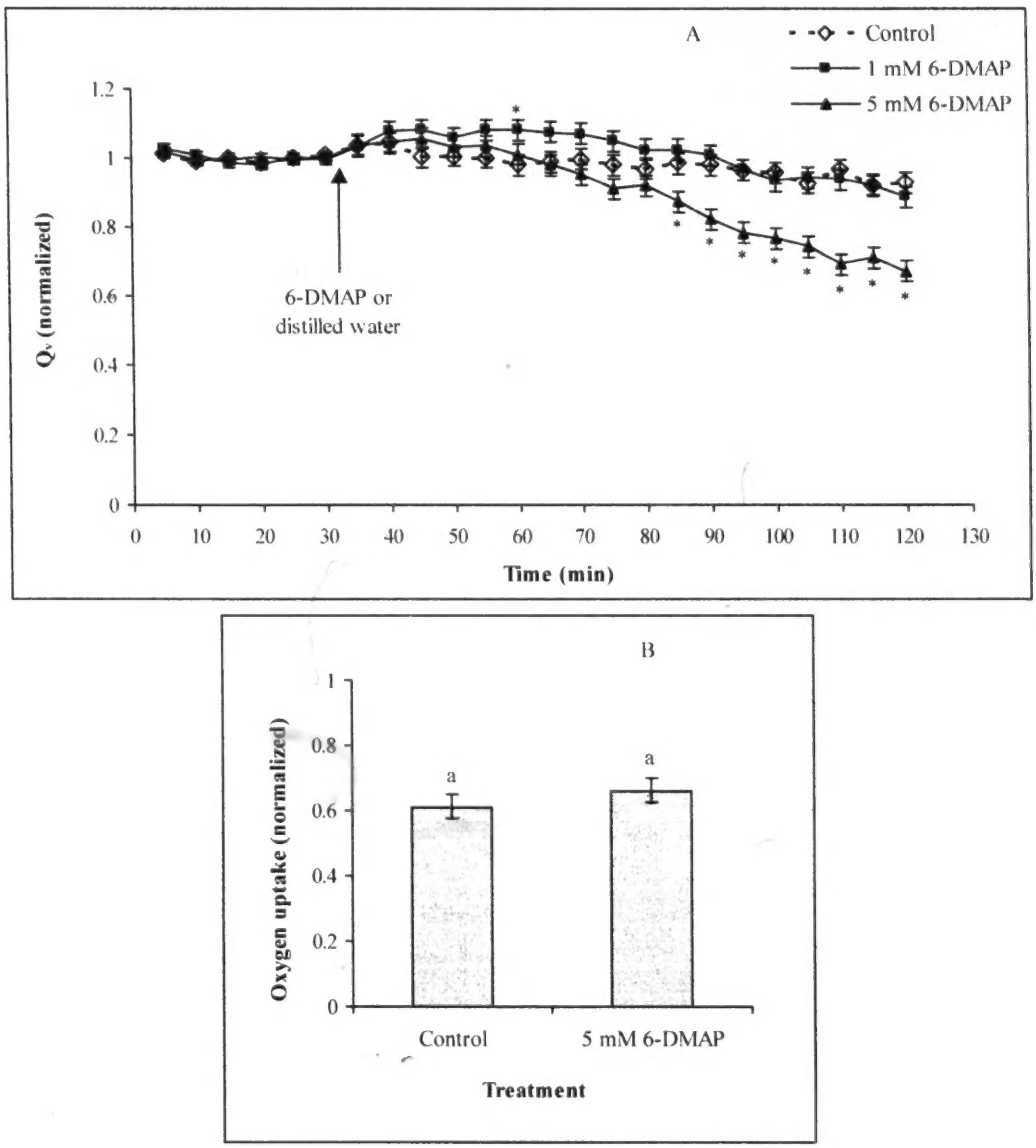


Figure 2.2 (A) Steady-state root water flow (Q_v) in response to different concentrations of the phosphorylation inhibitor 6-dimethyl aminopurine (6-DMAP). Data points represent least square means (n=6) ± SE. Arrow indicates the time of application of different chemical inhibitors. (* significantly different from the control at P≤0.05). (B) Root respiration in response to 1 mM 6-DMAP. Respiration rates obtained after 90 min treatment were normalized with the rates obtained before treatment application. Bars represent the least square means (n=6) ± SE.

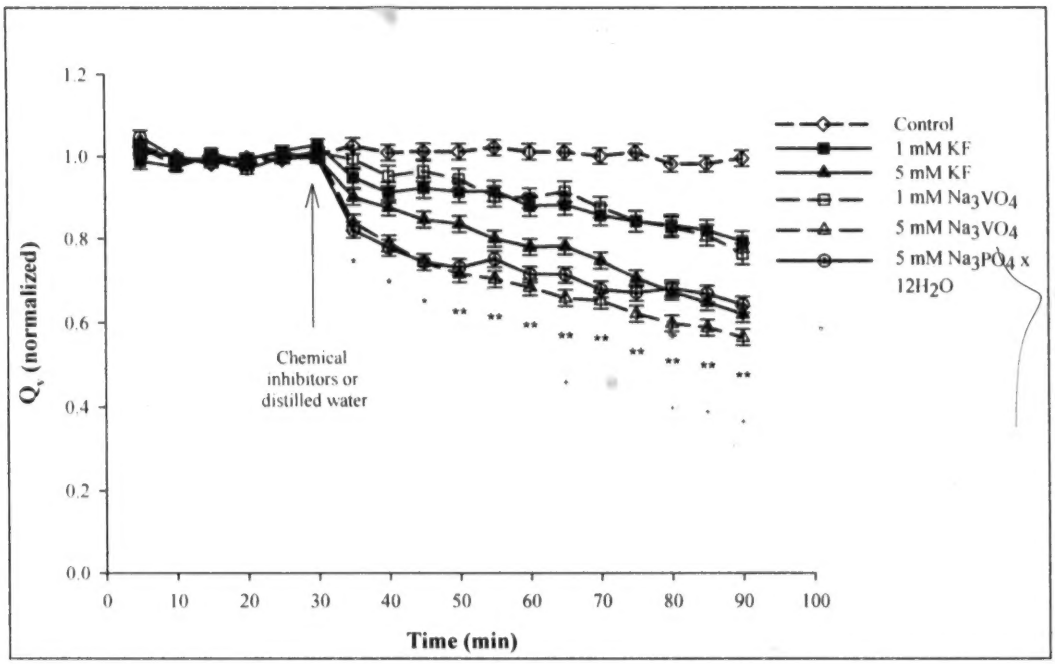


Figure 2.3 Changes in the root water flow rates (Q_v) over time in response to different concentrations of dephosphorylation inhibitors: KF, Na_3VO_4, and Na_3PO_4 x 12 H_2O. Data points represent adjusted least square means (n=6-10) ± SE. Arrow indicates the time of application of different chemical inhibitors. (* significantly different from the control for all treatments, except 1 mM Na_3VO_4, ** significantly different from the control for all treatments, + 5 mM Na_3VO_4 significantly different from the 5 mM Na_3PO_4 x 12 H_2O, $P \leq 0.05$).

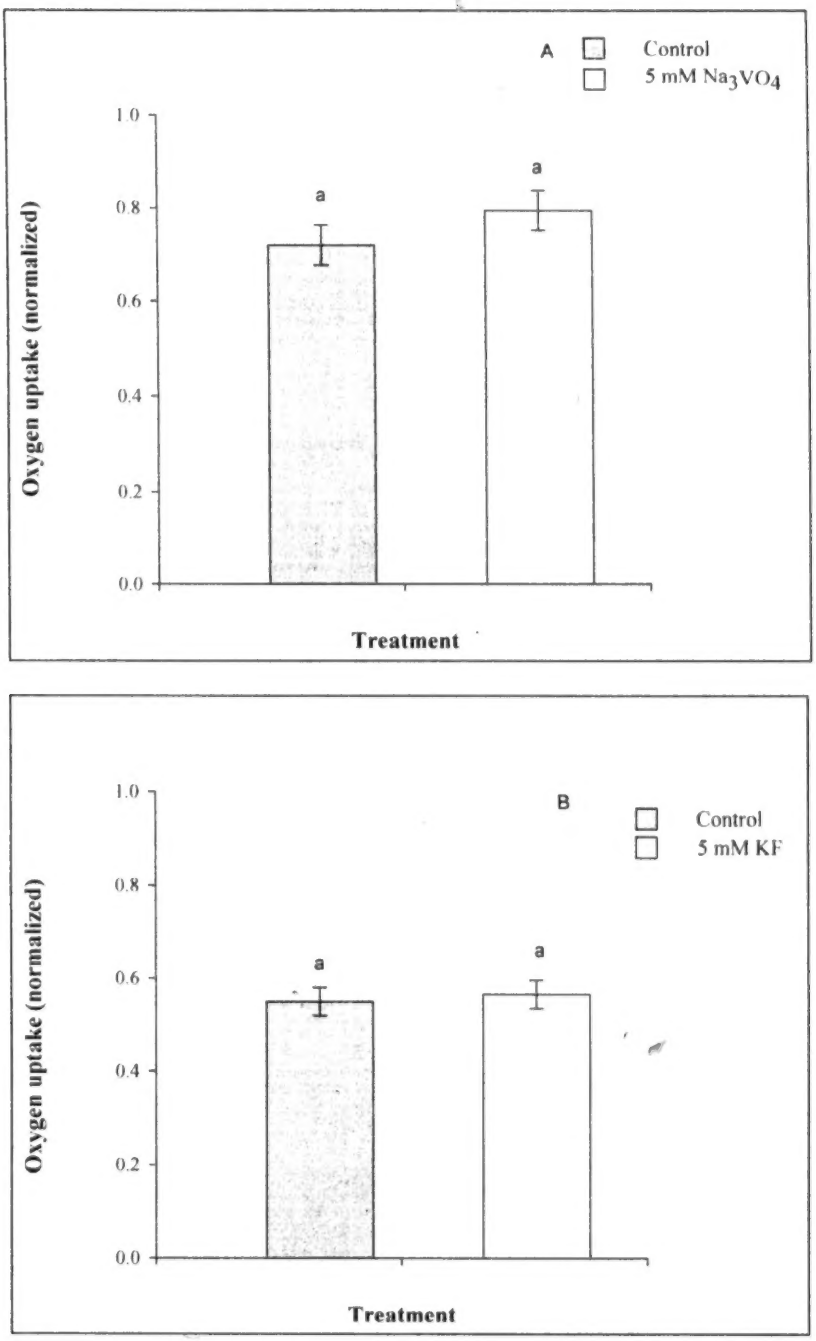


Figure 2.4 Root respiration measured as oxygen uptake for A) Na_3VO_4 and B) KF-treated roots. Rates obtained after 60 min treatment were normalized with the rates obtained before treatment application. Vertical bars represent adjusted least square means (n=8-9) ± SE. Bars with different letters are significantly different ($P \leq 0.05$).

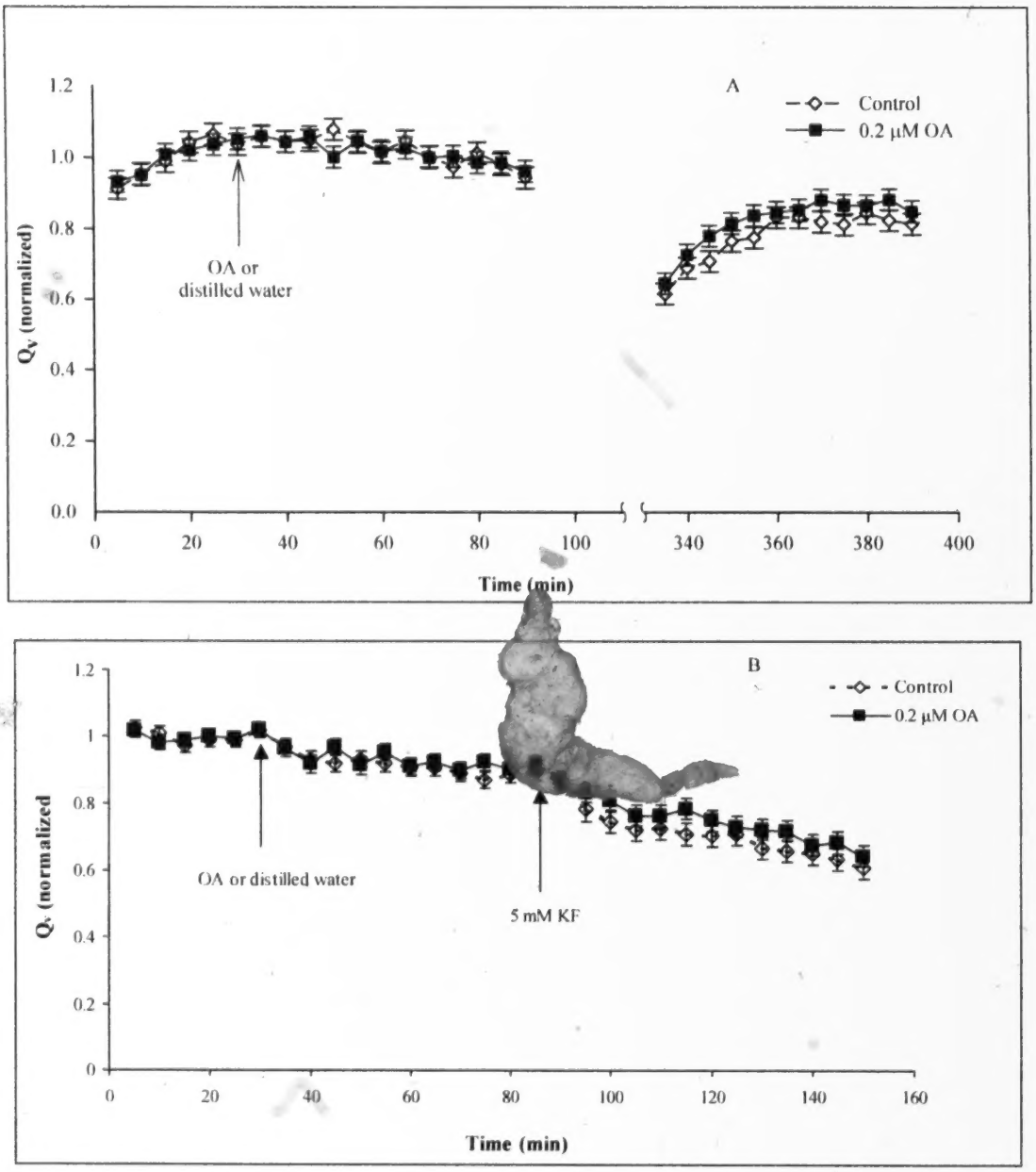
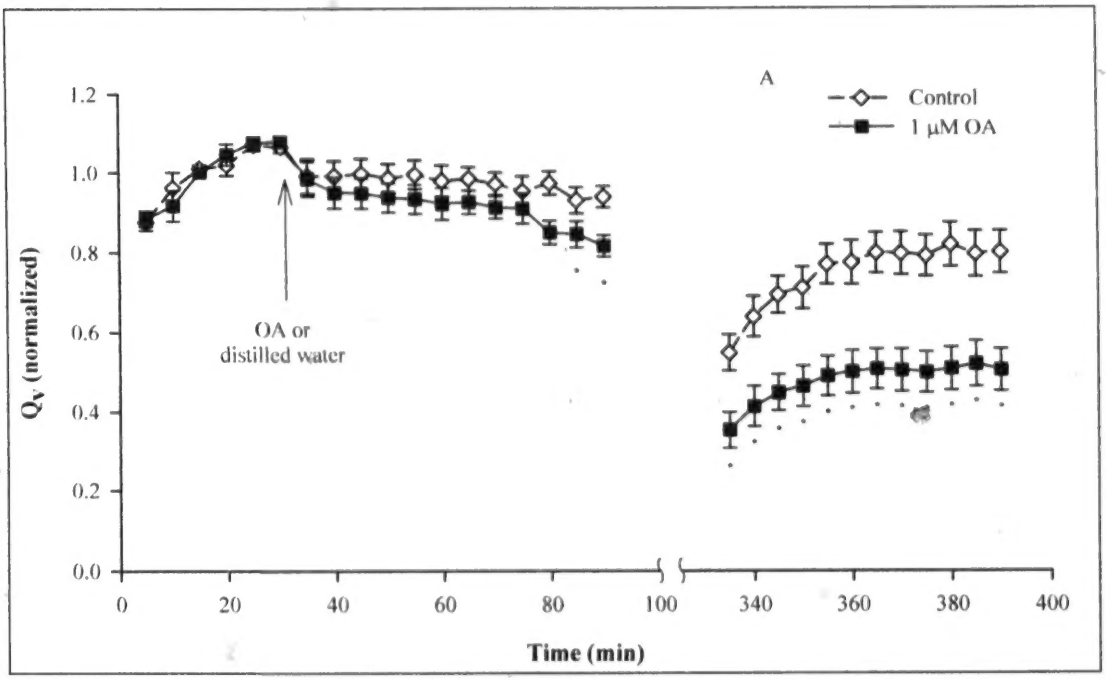


Figure 2.5 (A) Steady-state root water flow (Q_v) over time in response to 0.2 µM okadaic acid (OA). (B) Root water flow rates (Q_v) after OA and KF treatments. Data points represent adjusted least square means (n=6) ± SE. Arrow marks indicate the time of application of different chemical inhibitors.



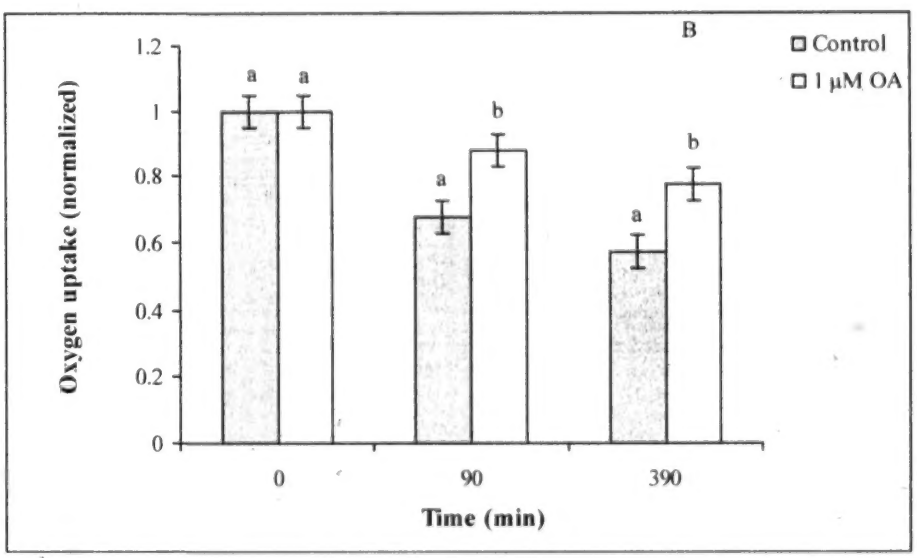


Figure 2.6 (A) Steady-state root water flow (Q_v) over time in response to 1 μM okadaic acid (OA). (B) Root respiration rates as oxygen uptake for the same roots. Data points represent adjusted least square means (n=6) ± SE. Arrow mark indicates the time the chemical inhibitor was applied. * significantly different from the control (P≤0.05). (B) Bars in the same group followed by different letters are significantly different (P≤0.05).

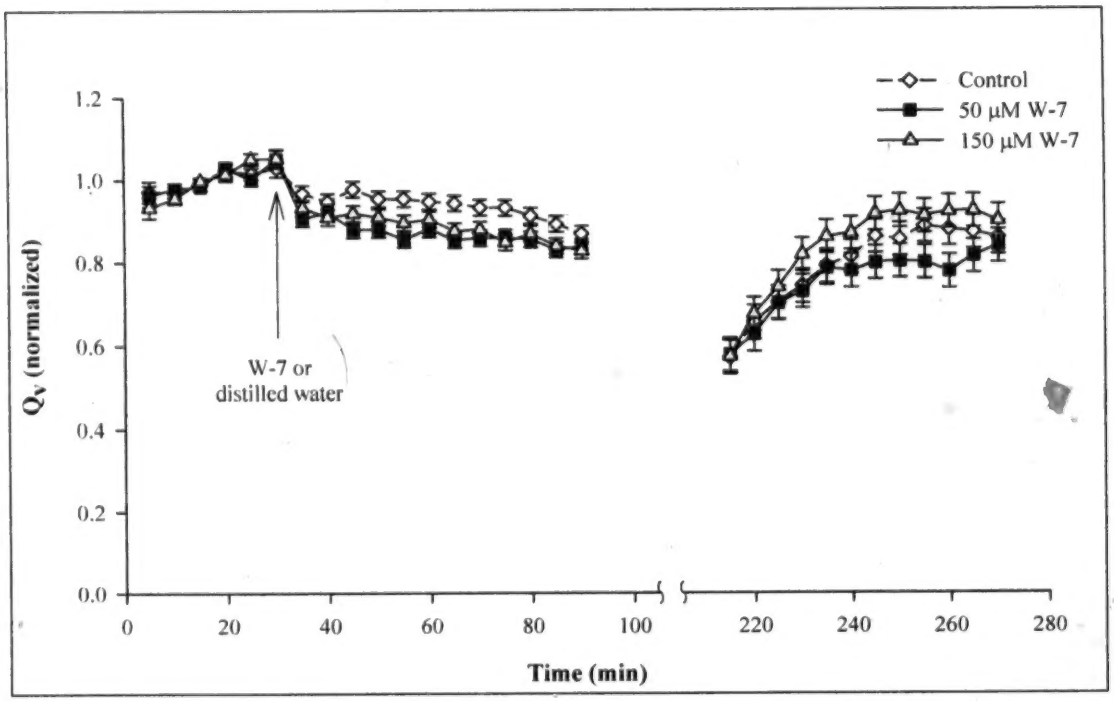


Figure 2.7 Steady-state root water flow (Q_v) over time in response to 50 μM and 150 μM N-(6-Aminohexyl)-5-chloro-1-naphtalene-sulfoamide (W-7). Arrow mark indicates the time of application of W-7. Data points represent adjusted least square means (n=6) ± SE

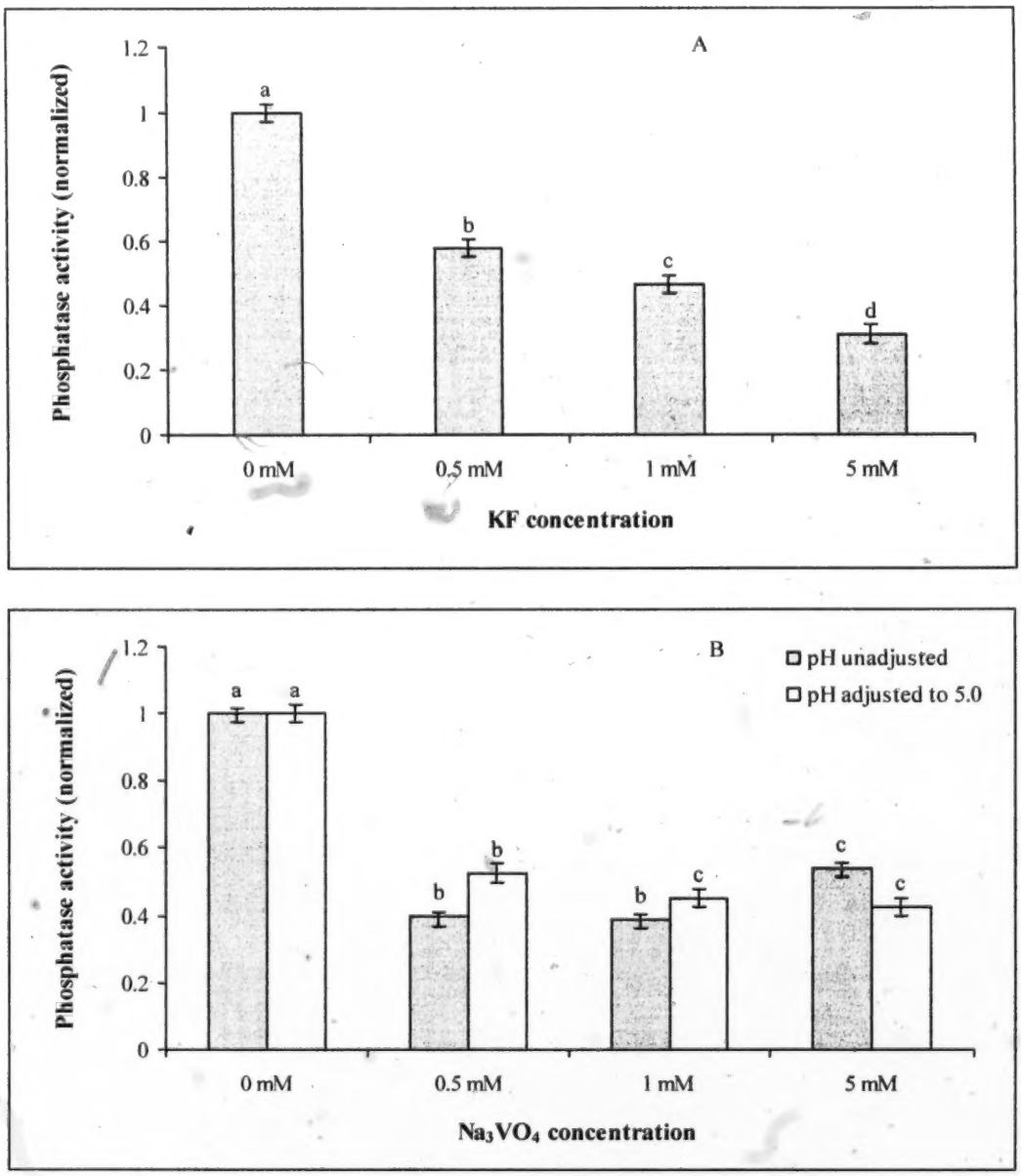
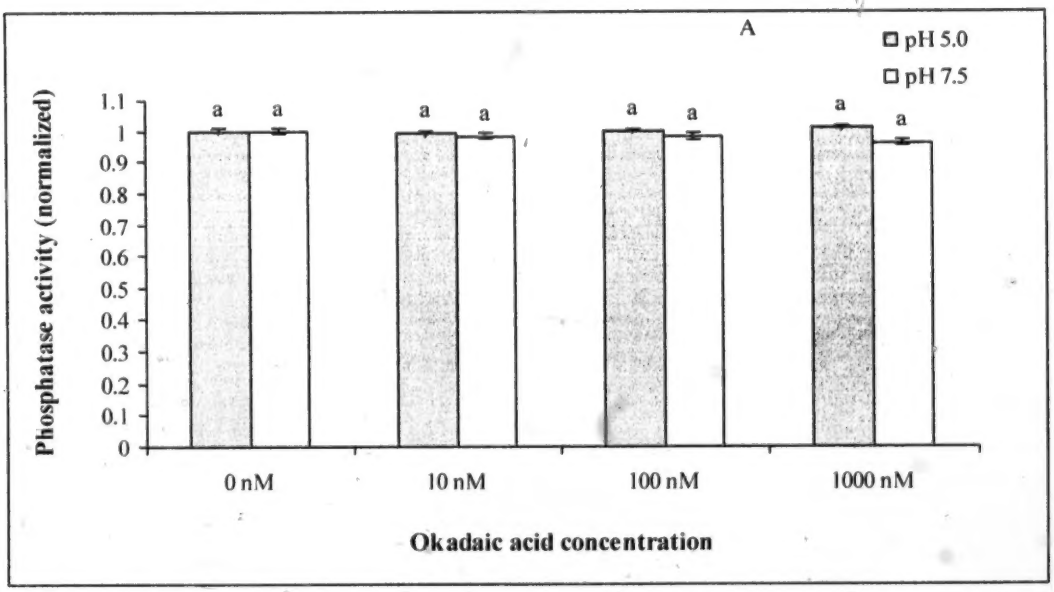


Figure 2.8 The *in vitro* acid phosphatase assay for different concentrations of KF (A) and Na_3VO_4 (B). Phosphatase activities were normalized with the corresponding values obtained for phosphatase activity for 0 mM. Adjusted least square means (n=6) ± SE are presented. Bars with different letters are significantly different from the corresponding 0 mM concentration ($P \leq 0.05$).



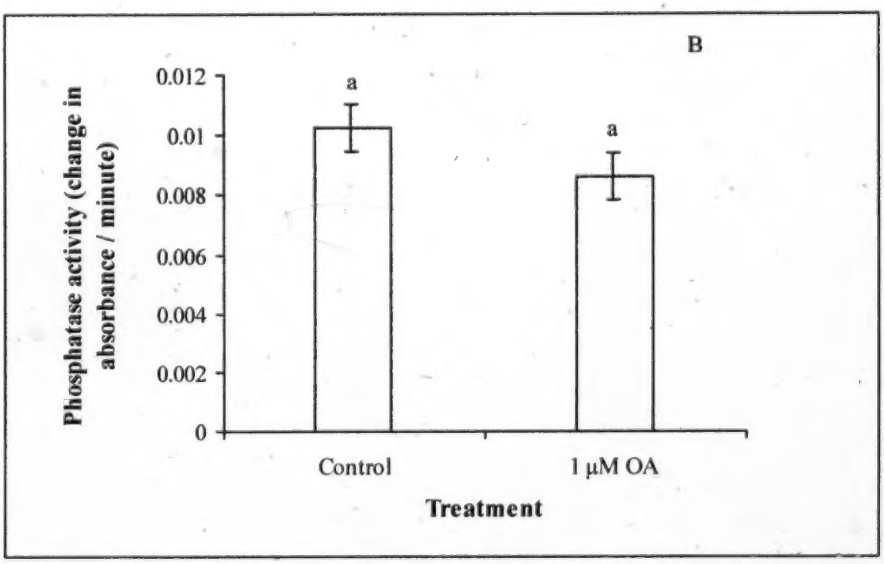


Figure 2.9 Acid phosphatase assay for (A) *in vitro* different concentrations of okadaic acid (OA) and (B) in extracts from control roots and roots treated with 1 µM OA for 5 hours. Adjusted least square means (n=6) ± SE are presented. Bars with different letters are significantly different from the corresponding control (P≤0.05).

References

Agrawal, G.K., Rakwal, R., and Jwa, N.-S. 2002. Cloning and characterization of a jasmonate inducible rice (*Oryza sativa* L.) peroxidase gene, OsPOX, against signaling molecules and certain inhibitors of kinase-signaling cascade(s). Plant Sci. 162: 49-58.

Ball, J.H., Williams, L., and Hall, J.L. 1987. Effect of SW26 and erythrosine B on ATPase activity an related processes in *Ricinus* cotyledons and cucumber hypocotyls. Plant Sci. 52: 1-5.

Ballantyne, D., and Glover, B.L. 1981. Chloroplast electron transport, protein and RNA in fluoride-treated pea shoots. Environ. Exp. Bot. 21: 83-88.

Baskin, T.I., and Wilson, J.E. 1997. Inhibitors of protein kinases and phosphatases alter root morphology and disorganize cortical microtubules. Plant Physiol. 113: 493-502.

Bigot, J., and Boucaud, J. 2000. Effects of Ca-signalling inhibitors on short-term cold-acclimation of hydraulic conductivity in roots of *Brassica rapa* plants. J. Plant Physiol. 157: 7-12.

Blackman, L.M., and Overall, R.L. 2001. Structure and function of plasmodesmata. Aust. J. Plant Physiol. 28: 709-727.

Brautigan, D.L., and Shriner, C.L. 1988. Methods to distinguish various types of protein phosphatase activity. Methods Enzym. 159: 339-346.

Carvajal, M., Cerdá, A., and Martinez, V. 2000. Does calcium ameliorate the negative effect of NaCl on melon root water transport by regulating aquaporin activity? New Phytol. 145: 439-447.

Carvajal, M., Martinez, V., and Alcaraz, C.F. 1999. Physiological function of water channels as affected by salinity in roots of paprika pepper. Physiol. Plant. 105: 95-101.

Cazalé, A.-C., Droillard, M.-J., Wilson, C., Heberle-Bors, E., Barbier-Brygoo, H., and Laurière, C. 1999. MAP kinase activation by hypoosmotic stress of tobacco cell suspension: towards the oxidative burst response? Plant J. 19(3): 297-307.

Chandok, M.R., and Sopory, S.K. 1996. Phosphorylation/dephosphorylation steps are the key events in the phytochrome-mediated enhancement of nitrate reductase mRNA levels and enzyme activity in maize. Mol. Gen. Genet. 251: 599-608.

Chandok, M.R., and Sopory, S.K. 1998. ZmcPCK70, a protein kinase C-type enzyme from maize. J. Biol. Chem. 273(30): 19235-19242.

Chen, H.-J., Hou, W.-C., Kuc, J., and Lin, Y.-H. 2001. Ca^{2+}-dependent and Ca^{2+}-independent excretion modes os salicylic acid in tobacco cell suspension culture. J. Exp. Bot. 52(359): 1219-1226.

Chen, T.-H., Hsu, C.-S., Tsai, P.-J., Ho, Y.-F., Lin, N.-S. 2001. Heterotrimeric G-protein and signal transduction in the nematode-trapping fungus *Arthrobotrys dactyloides*. Planta 212: 858-863.

Chou, C.-L., Yip, K.-P., Michea, L., Kador, K., Ferraris, J.D., Wade, J.B., and Knepper, M.A. 2000. Regulation of aquaporin-2 trafficking by vasopresin in the renal collecting duct. J. Biol. Chem. 275(47): 36839-36846.

Clarkson, D.T., Carvajal, M., Henzler, T., Waterhouse, R.N., Smyth, A.J., Cooke, D.T., and Steudle, E. 2000. Root hydraulic conductance: diurnal aquaporin expression and the effects of nutrient stress. J. Exp. Bot. 51(342): 61-70.

Cohen, P., Holmes, C.F.B., and Tsukitani, Y. 1990. Okadaic acid: a new probe for the study of cellular regulation. TIBS 15: 98-102.

Deshpande, M.V., O'Donnell, R., and Gooday, G.W. 1997. Regulation of chitin synthase activity in the dimorphic fungus *Benjaminiella poitrasii* by external osmotic pressure. FEMS Microbiol. Letters 152: 327-332.

Deswal, R., Pandey, G.K., Chandok, M.R., Yadav, N., Bhattacharya, A., and Sopory, S.K. 2000. A novel protein kinase from *Brassica juncea* stimulated by a protozoan calcium binding protein. Eur. J. Biochem. 267: 3181-3188.

Epstein, E. 1972. Mineral nutrition of Plants: Priciples and Perspectives. John Wiley & Sons, London.

Estruch, J.J., Kadwell, S., Merlin, E., and Crossland, L. 1994. Cloning and characterization of a maize pollen-specific calcium-dependent calmodulin-independent protein kinase. Proc. Natl. Acad. Sci. USA 91 (19): 8837-8841.

Fleurat-Lessard, P., Frangne, N., Maeshima, M., Ratajczak, R., Bonnemain, J.-L., and Martinoia, E. 1997. Increased expression of vacuolar aquaporin and H^+-ATPase related to motor cell function in *Mimosa pudica* L. Plant Physiol. 114: 837-834.

Gerbeau, P., Amodeo, G., Hnezler, T., Santoni, V., Ripoche, P. and Maurel, C. 2002. The water permeability of *Arabidopsis* plasma membrane is regulated by divalent cations and pH. Plant J. 30(1): 71-81.

Gerendás, J., and Ratcliffe, R.G. 2002. Root pH regulation. In Wisel, Y., Eshel, A., and Kafkafi, U. Eds. Plant Roots: The Hidden Half. 3rd ed. Dekker, M., New York. pp: 553-570.

Gout, E., Bligny, R., and Doucet, R. 1992. Regulation of intracellular pH values in higher plant cells. Carbon-13 and phosphorus-31 nuclear magnetic resonance studies. J. Biol. Chem. 267(20): 13903-13909.

Graham, D.L., Eccleston, J.F., Chung, C.-W., and Lowe, P.N. 1999. Magnesium fluoride-dependent binding of small G proteins to their GTPase-activating proteins. Biochem. 38: 14981-14987.

Gunsé, B., Poschenrieder, C., and Barceló, J. 1997. Water transport properties of roots and root cortical cells in proton- and Al-sensitive maize varieties. Plant Physiol. 113: 595-602.

Hardie, D.G., Haystead, T.A.J., and Sim, A.T.R. 1991. Use of okadaic acid to inhibit protein phosphatases in intact cells. Methods Enzym. 201: 469-482.

Harvengt, P., Vlerick, A., Fuks, B., Wattiez, R., Ruysschaert, J.M., and Homble, F. 2000. Lentil seed aquaporins form a hetero-oligomer which is phosphorylated by a Mg^{2+}-dependent and Ca^{2+}-regulated kinase. Biochem. J. 352: 183-190.

Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. 1984. Isoquinolinesulfoamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochem. 23: 5036-

5041.

Hidaka, H., and Kobayashi, R. 1993. Use of protein (serine/threonine) kinase activators and inhibitors to study protein phosphorylation in intact cells. In DG Hardie Eds. Protein posphorylation, a practical approach. Oxford University Press, Oxford, UK, pp. 87-107.

Hidaka, H., Watanabe, M., and Kabayasho, R., 1991. Properties and use of H-series compounds as protein kinase inhibitors. Methods Enzym. 201: 328-338.

Hidalgo, A., Navas, P., and Garcia-Herdugo, G. 1988. Growth inhibition induced by vanadate in onion roots. Environ. Exp. Bot. 28(2): 131-136.

Huang, J.Z., Hardin, S.C., and Hubert, S.C. 2001. Identification of a novel phosphorylation motif for CDPKs: phosphorylation of synthetic peptides lacking basic residues at P-3/P-4. Archi. Biochem. Biophys. 393(1): 61-66.

Javot, H., and Maurel, C. 2002. The role of aquaporins in root water uptake. Ann. Bot. 90: 301-313.

Johansson, I., Karlsson, M., Shukla, V.K., Chrispeels, M.J., Larsson, C., and Kjellbom, P. 1998. Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. Plant Cell 10: 451-460.

Johansson, I., Larsson, C., Ek, B., and Kjellbom, P. 1996. The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^{2+} and apoplastic water potential. The Plant Cell 8: 1181-1191.

Kamaluddin, M., and Zwiazek, J.J. 2001. Metabolic inhibition of root water flow in red-osier dogwood (*Cornus stolonifera*) seedlings. J. Exp. Bot. 52 (357): 739-745.

Kamaluddin, M., and Zwiazek, J.J. 2002. Ethylene enhances water transport in hypoxic aspen. Plant Physiol. 128: 1-8.

Kamaluddin, M., and Zwiazek, J.J. 2003. Inhibition of root water transport by fluoride affects leaf expansion and gas exchange in aspen (*Populus tremuloides* Michx.) seedlings. Physiol. Plant. (in press).

Kjelbom, P., Larsson, C., Johansson, I., Karlsson, M., and Johanson, U. 1999. Aquaporins and water homeostasis in plants. T. Plant Sci. 4(8): 308-314.

Kutuzov, M.A., and Andreeva, A.V. 2001. Noncompetitiv inhibition of plant protein Ser/Thr phosphatase PP7 by phosphate. Biochem. Biophys. Res. Com. 283: 93-96.

Lee, Y., and Assmann, S.M. 1991. Diacylglycerols induce both ion pumping in patch-clamped guard-cell protoplasts and opening of intact stomata. Proc. Natl. Acad. Sci. USA 88: 2127-2131.

Leitner-Dagan, Y., and Weiss, D. 1999. Ca^{2+}, calmodulin, and protein dephosphorylation are required for GA-induced expression in petunia corolla. Physiol. Plant. 105: 116-121.

Li, W.G., and Komatsu, S. 2000. Cold stress-induced calcium-dependent protein kinase(s) in rice (*Oryza sativa* L.) seddling stem tissues. Theor. Appl. Genet. 101: 355-363.

Luan, S. 2000. Protein phosphatases: structure, regulation, and function. In M Kreis, JC Walker & JA Callow Eds. Plant protein kinases: advances in botanical research incorporating advances in plant

pathology. Academic Press, London, pp. 67-107.

MacKintosh, C., Cohen, P. 1989. Indentification of high levels of type 1 and type 2A protein phosphatases in higher plants. Biochem. J. 262: 335-339.

Maggio, A., and Joly, R.J. 1995. Effects of mercuric chloride on the conductivity of tomato root system. Plant Physiol. 109: 331-335.

Markhart III, A.H., Fiscus, E.L., Naylor, A.W., and Kramer, P.J. 1979. Effect of temperature on water and ion transport in soybean and broccoli systems. Plant Physiol. 64: 83-87.

Markhart, A.H., and Smit, B. 1990. Measurement of root hydraulic conductance. HortSci. 25: 282-287.

Martinez-Ballesta, M.C., Martinez, V., and Carvajal, M. 2000. Regulation of water channel activity in whole roots and in protoplasts from roots of melon plants grown under saline conditions. Aust. J. Plant Physiol. 27: 685-691.

Mathieu, Y., Snachez, F.J., Droillard, M.-J., Lapous, D., Laurière, C., and Guern, J. 1996. Involvement of protein phosphorylation in the early steps of transduction of the oligogalacturonide signal in tobacco cell. Plant Physiol. Biochem. 34(3): 399-408.

Mathooko, F.M., Inaba, A., and Nakamura, R. 1998. Characterization of carbon dioxide stress-induced ethylene biosynthesis in cucumber (*Cucumis sativus* L.) fruit. Plant Cell Physiol. 39(3): 285-293.

Meychik, N.R., and Yermakov, I.P. 2001. Swelling of root cell walls as an indicator of their functional state. Biochem. 66(2): 178-187.

Michelet, B., and Boutry, M. 1996. Proton-translocating ATPases of the plasma membrane: biological functions, biochemistry and molecular genetics. In M Smallwood, JP Knox, and DJ Bowels Eds. Membranes: Specialized functions in plants. BIOS Scientific Publishers, Oxford, UK, pp: 261-279.

Mizuno, K. 1992. Induction of cold stability of microtubules in cultured tobacco cells. Plant Physiol. 100: 740-748.

Mizuno, K. 1994. Inhibition of gibberellin-induced elongation, reorientation of cotical microtubules and change of isoform of tubulin in epicotyl segments of azuki bean by protein kinase inhibitors. Plant Cell Physiol. 35(8): 1149-1157.

Mori, I.C., Uozumi, N., and Muto, S. 2000. Phosphorylation of the inward-rectifying potassium channel KAT1 by ABR kinase in *Vicia* guard cells. Plant Cell Physiol. 41(7): 850-856.

Morsomme, P., and Boutry, M. 2000. The plant plasma membrane H^+-ATPase: structure, function and regulation. Biochim. Biophys. Acta 1465: 1-16.

Neant, I., and Guerrier, P., 1988. 6-Dimethylaminopurine blocks starfish oocyte maturation by inhibiting a relevant protein kinase activity. Exp. Cell Res. 176: 68-79.

Németh-Cahalan, K., and Hall, J.K. 2000. pH and calcium regulate the water permeability of aquaporin 0. J. Biol. Chem. 275(10): 6777-6782.

Oparka, K.J., and Prior, D.A.M. 1992. Direct evidence for pressure-generated closure of palsmodesmata. Plant J. 2(5): 741-750.

Palme, K. 1996.A role for the heterotrimeric G-protein switch in higher plants. In M Smallwood, JP Knox,

and DJ Bowels Eds. Membranes: Specialized functions in plants. BIOS Scientific Publishers, Oxford, UK, pp: 151-163.

Park, M.H., and Chae, Q. 1990. Intracellular protein phosphorylation in oat (*Avena sativa* L.) protoplasts by phytochrome action: involvement of protein kinase C. Biochem. Biophys. Res. Comm. 69(3): 1185-1190.

Pedersen, O. 1993. Long-distance water transport in aquatic plants. Plant Physiol. 103: 1369-1375.

Rakowski, K.J. 1997. Hydrogen fluoride effects on plasma membrane composition and ATPase activity in needles on white pine (*Pinus strobus*) seedlings pretreated with 12 h photoperiod. Trees 11: 248-253.

Rakowski, K.J., and Zwiazek, J.J., and Sumner, M.J. 1995. Hydrogen fluoride effects on plasma membrane composition, ATPase activity and cell structure in needles of eastern white pine (*Pinus strobus*) seedlings. Trees 9: 190-194.

Rakwal, R., Agrawal, G.K., and Yonekura, M. 2001. Light-dependent induction of OsPR10 in rice (*Oryza sativa* L.) seedlins by the global stress siganling molecules jasmonic acid and protein phosphatase 2A inhibitors. Plant Sci. 161: 469-479.

Rebhun, L.I., White, D., Sander, G., and Ivy, N. 1973. Cleavage inhibition in marine eggs by puromycin and 6-dimethylaminopurine. Exp. Cell Res. 77: 312-318.

Roberts, D.M., and Harmon, A.C. 1992. Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43: 375-414.

Roberts, D.M., Lukas, T.J., and Watterson, D.M. 1986. Structure, function, and mechanism of action of calmodulin. CRC Critical Rev. Plant Sci. 4(4): 311-339.

Romeis Tina, Piedras, P., and Jones, J.D.G. 2000. Resistance gene-dependant activation of a calcium-dependent protein kinase in the plant defense response. Plant Cell 12: 803-815.

Rugini, E., and Caricato, G. 1995. Somatic embryogenesis and plant recovery from mature tissues of olive cultivars (*Olea europaea* L.) "Canino" and "Moraiolo". Plant Cell Rep. 14: 257-260.

Sakano, K., Yazaki, Y., and Mimura, T. 1992. Cytoplasmic acidificatio induced by inorganic phosphate uptake in suspension cultured *Cantharanthus roseus* cells. Plant Physiol. 99: 672-680.

Sanders, D., Brownlee, C., and Harper, J.F. 1999. Communicating with calcium. Plant Cell 11: 691-706.

SAS 2000. SAS/STAT User's Guide (Version 8). SAS Inst. Inc., Cary, NC.

Shiratake, K., Goto, H., Maeshima, M., and Yamaki, S. 2001. Fluctuations in the protein levels of H^+-pumps and water channels of the tonoplst and plasma membrane during grape berry development. J. Japan. Soc. Hort. Sci. 70(3): 287-293.

Sopory, S.K., and Munshi, M. 1998. Protein kinases and phosphatases and their role in cellular signaling in plants. CRC Plant Sci. 17(3): 245-318.

Steudle, E. 2000. Water uptake by the plant roots: an integration of views. Plant and Soil 226: 45-56.

Steudle, E., and Frensch, J. 1996. Water transport in plants: role of the apoplast. Plant and Soil 187: 67-79.

Struglics, A., Fredlund, K.M., Kostantinov, Y.M., Allen, J.F., and Møller, I.M. 2000. Protein

phosphorylation/dephosphorylation in the inner membrane of potato tuber mitochondria. FEBS Letters 475: 213-217.

Szöllösi, M.S., Kubiak, J.Z., Debey, P., dePennart, H., Szöllösi, D., and Maro, B. 1993. Inhibition of protein kinases by 6-dymethylaminopurine accelerates the transition to interphase in activated mouse oocytes. J. Cell Sci. 104: 861-872.

Tamaoki, T. 1991. Use and specificity of staurosporine, UNC-01, and calphosptin C as protein kinase inhibitors. Methods Enzym. 201: 340-347.

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F. 1986. Stauroporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. Biochem. Biophys. Res. Comm. 135(2): 397-402.

Tazawa, M., Ohkuma, E., Shibasaka, M., and Nakashima, S. 1997. Mercurial-sensitive water transport in barley roots. J. Plant Res. 110: 435-442.

Tazawa, M., Sutou, E., and Shibasaka, M. 2001. Onion root water transport sensitive to water channel and K^+ channel inhibitor. Plant Cell Physiol. 42(1): 28-36.

Valenti, G., Procino, G., Carmosino, M., Frigeri, A., Mannuci, R., Nicoletti, I., and Svelto, M. 2000. The phosphatase inhibitor okadaic acid induces AQP2 translocation independently from AQP2 phosphorylation in renal collecting duct cells. J. Cell Sci. 113: 1985-1992.

van Balkom, B.W.M., Savelkoul, P.J.M., Markovich, D., Hofma, E., Nielsen, S., van der Sluijs, P., and Deen, P.M.T. 2002. The role of putative phosphorylation sites in the targeting and shuttling of the aquaporin-2 water channel. J. Biol. Chem. 277(44): 41473-41479.

Vitrac, X., Larronde, F., Krisa, S., Decendit, A., Deffieux, G., and Merillon, J.-M. 2000. Sugar sensing and Ca^{2+}-calmodulin requirement in *Vitis vinifera* cells producing anthocyanins. Phytochem. 53: 659-665.

Wan, X., Landhäusser, S.M., Zwiazek, J.J., and Lieffers, V.J. 1999. Root water flow and growth of aspen (*Populus tremuloides*) at low root temperatures. Tree Physiol. 19: 879-884.

Wan, X., and Zwiazek, J.J. 1999. Mercuric chloride effects on root water transport in aspen seedlings. Plant Physiol. 121: 939-946.

Widell, S., and Larsson, C. 1990. A critical evaluation of markers used in plasma membrane purification. In C. Larsson and I.M. Moller Eds. The Plant Plasma Membrane. Sprin-Verlag, Berlin, Germany, pp: 16-43.

Yan, F., Schubert, S., and Mengel, K. 1992. Effect of low root medium pH on net proton release, root respiration, and root growth of corn (*Zea mays* L.) and broad bean (*Vicia faba* L.). Plant Physiol. 99: 415-421.

Yang, G., and Komatsu, S. 2000. Involvement of calcium-dependent protein kinase in rice (*Oryza sativa* L.) lamina inclination caused by brassinolide. Plant Cell Physiol. 41(11): 1243-1250.

Yasui, M., Hazama, A., Known, T.-H., Nielsen, S., Guggino, W.B., and Agre, P. 1999. Rapid gating and anion permeability of an intracellular aquaporin. Nature 402: 184-187.

Yoshida, S., and Anraku, Y. 2000. Characterization of staurosporine-sensitive mutants of *Saccharomyces cerevisiae*: vacuolar functions affect staurosporine sensitivity. Mol. Gen. Genet. 263: 877-888.

Zhang, W.H., and Tyreman, S.D. 1991. Effect of low O_2 concentration on azide on hydraulic conductivity and osmotic volume of the cortical cells of wheat roots. Aust. J. Plant Physiol. 18: 603-613.

Zhang, W.H., and Tyreman, S.D. 1999. Inhibition of water channels by $HgCl_2$ in intact wheat root cells. Plant Physiol. 120: 849-857.

Zieslin, N., and Abolitz, M. 1994. Leakage of phenolic compunds from plant roots: effects of pH, Ca^{2+} and NaCl. Scientia Horticulturae 58: 303-314.

Zwiazek, J.J., and Shay, J.M. 1988. Sodium fluoride induced metabolic changes in jack pine seedlings. II. Effects on growth, acid phosphatase, cytokinins, and pools of soluble proteins, amino acids, and organic acids. Can. J. For. Res. 18: 1311-1317.

Chapter 3. Cycloheximide inhibits root water flow and stomatal conductance in aspen (*Populus tremuloides*) seedlings

3.1. Introduction

The variability of root water uptake has been known for a long time (Fiscus 1975, Passioura 1988, Steudle and French 1996, Steudle 2000). With the development of the composite model for root water uptake, explanations for root hydraulic conductivity (L_p) variability started to emerge. The radial water transfer across roots accounts for the greatest resistance in water movement within plants (Steudle and Peterson 1998). There are three pathways for radial water transport across living root tissues: 1) through the cell walls (apoplastic path), 2) along the symplasm through plasmodesmata (symplastic path), and 3) across cell membranes (transcellular path) (Steudle and Peterson 1998, Javot and Maurel 2002). The discovery of aquaporins and their diversity within plants has provided a key factor for understanding water transport processes across membranes. Root water flow changes rapidly in response to environmental changes (Nobel et al. 1990, Else et al. 1995, Lu and Newman 1999, Carvajal et al. 1999, Clarkson et al. 2000) and there is growing evidence that aquaporins are involved in this response (Javot and Maurel 2002). However, according to the composite model, at the root and whole plant levels as well as under normal transpiring conditions (hydrostatic gradients), water is thought to move mostly through the apoplast (Steudle 2000). Some questions of this model have been raised since the apoplastic barriers in the endodermis and exodermis were initially thought to be relatively impermeable to water (Steudle and Peterson 1998, Zimmerman et

al. 2000, Zimmermann and Steudle 1998). It has also been questioned whether the proposed model can explain the capacity of roots to alter L_p over a short time in response to many stimuli and in the absence of changes in root structure (Magio and Joly 1995, Wan and Zwiazek 1999, Martinez-Balesta et al. 2000, Kamaluddin and Zwiazek 2001, Carvajal et al. 2000). Therefore, at least in some plant species, the cell-to-cell pathway may be playing a more important role than it was initially thought (Javot and Maurel 2002). The contribution of water channels to the overall water transport has been mostly tested with $HgCl_2$, despite its non-specific and toxic effects (Tazawa et al. 1997, Wan and Zwiazek 1999, Barrowclough et al. 2000, Javot and Maurel 2002). Mercuric chloride decreases the water uptake by direct blocking the aquaporin protein (Barrowclough et al. 2000).

At the membrane level, the total water flow through aquaporins depends on the density (abundance) of aquaporins in the membrane and on their activity (Chaumont et al. 2000, Tyreman et al. 2002). While phosphorylation and dephosphorylation or other gating mechanisms that regulate water permeability of aquaporins *in situ* have been proposed (Maurel et al. 1995, Johansson et al. 1996, Harvengt et al. 2000), little attention has been paid to the turnover rate of aquaporins in the membranes (Suga et al. 2001). The expression of aquaporin genes is regulated in an organ- and stage-specific manner (Suga et al. 2001). Henzler et al. (1999) showed that in the roots of *Lotus japonicus*, the diurnal variation in root L_p is correlated with the diurnal fluctuation of PIP1-type aquaporin in the plasma membrane. Water fluxes across the plasma membrane could also be regulated through differential expression of the various aquaporin isoforms (Fotidias et al. 2001). Although there have been several studies that investigated the aquaporin abundance

(Kirch et al. 2000, Fotidias et al. 2001), the studies have not assessed the significance of translational regulation of aquaporins for water relations at the whole plant level.

In the present study, I used cycloheximide, a known protein synthesis inhibitor, to assess the importance of protein synthesis in plant water relations. Cycloheximide inhibits both peptide initiation and extension (O'Brig et al. 1971). Cycloheximide was found to be more effective in inhibiting the microsomal protein than the total protein content in wheat (Zhang et al. 1995) and it was successfully used to study water transport in mammalian and plant cells and protoplasts (Katsura et al. 1996, Yamamoto et al. 2001, Moshelion et al. 2002). In the present study, I examined the effect of cycloheximide on root water transport in aspen (*Populus tremuloides* Michx.) seedlings. I used the pressure-flux method to measure the steady-state root water flow (Q_v) and root hydraulic conductivity (L_p) in roots of plants growing in solution culture. I also used the apoplastic dye trisodium 3-hydroxy-5,8,10-pyrenetrisulphonate (PTS_3) (Kamaluddin and Zwiazek 2001) to examine changes in the proportion of apoplastic root water flow in cycloheximide-treated plants. Since root respiration (Kamaluddin and Zwiazek 2001), stomatal conductance (Else et al. 1995, Wan and Zwiazek 1999), and phosphorylation and dephosphorylation events (Johansson et al. 1998) have been linked to aquaporin activity, I measured root respiration, acid phosphatase activity, and leaf stomatal conductance to examine possible indirect effects of cycloheximide on aquaporin function. I studied the hypotheses that: 1) cycloheximide would inhibit the synthesis of aquaporins and inhibit water flux, and 2) since gene expression and protein synthesis processes require longer time to express, the *de novo* aquaporin synthesis is not sufficiently rapid to serve as a primary regulation mechanism for root water flow.

3.2. Materials and Methods

3.2.1. Plant material

Aspen (*Populus tremuloides* Michx.) seeds were collected from Edmonton, Alberta, Canada. The seeds were germinated on sand in Petri dishes and when 5 days old, the seedlings were transferred to styrofoam containers (160/60 ml Super Block, Beaver Plastic, Edmonton, AB) filled with a mixture of sand, peat, and perlite (3:3:1, by volume). The seedlings were grown for 6 weeks in a growth room set to a 16-h photoperiod, photosynthetic photon flux density of 300 μmol m^{-2} s^{-1}, 22°C/18°C day/night temperatures, and a constant relative humidity of approximately 70%. The seedlings were bottom-watered and fertilized twice a week with 1% (w/v) solution of N-P-K (20-20-20) commercial fertilizer.

When six-weeks old, seedling roots were gently washed free of soil with distilled water and the seedlings were placed in 11-l plastic containers (10 seedlings per container) with their roots immersed in aerated full-strength modified Hoagland's mineral solution (Epstein 1972). After two weeks, the seedlings were removed from the containers and assigned to experimental treatments.

3.2.2. Steady-state root water flow rate (Q_v)

The steady-state root water flow rate (Q_v) was measured using the hydrostatic pressure method as previously described (Wan and Zwiazek 1999, Kamaluddin and

Zwiazek 2001). Whole root systems were excised 1 cm above the root collar and immersed in 250 ml distilled water in polyvinyl chloride containers that were placed inside pressure chambers (PMS Instruments, Corvallis, OR, U.S.A.). The cut stem protruded through a lid and was used to collect xylem sap. The bathing solution was kept continuously stirred with a magnetic stirrer. Pressure inside the pressure chamber was gradually increased and maintained constant at 0.3 MPa throughout the experiment. A graduated pipette (1 ml) was connected through a small piece of rubber tubing (1-2 mm diameter) to the cut stem and the volume of water expressed from the root system was monitored over time. Readings were taken at five minute intervals to calculate Q_v. The initial water flow was monitored for 30 min. Subsequently, the pressure was released and the root medium was replaced with fresh distilled water for control roots or with solutions containing different concentrations of cycloheximide (CHX). Cycloheximide (reagent grade, Sigma, St. Louis, MO, U.S.A.) solutions (0.25 mM, 0.5 mM, and 1 mM) were prepared by dissolving directly in distilled water. The roots were then pressurized again at 0.3 MPa and Q_v recorded for 90 min. For each root system, the mean flow rate over the first 30 min before adding CHX was used to normalize the data. Six replicates were used for each treatment. At the end of the Q_v measurements, roots were immediately frozen in liquid nitrogen and placed at -85°C before protein extraction.

3.2.3. Root respiration and Q_v measurements

In a different experiment, root respiration was measured as oxygen uptake using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.)

(Kamaluddin and Zwiazek 2002). The oxygen probe and the root system were enclosed in an airtight cylinder containing 340 ml aerated distilled water (approximately 7-8 mg l^{-1} initial O_2 concentration). The solution was kept continuously stirred with a magnetic stirrer. The oxygen content was recorded every 5 min, for 25 min. Root O_2 uptake was calculated as the slope of the regression line of oxygen concentration versus time. After the initial root respiration was measured, roots were pressurized for Q_v measurements. Then, Q_v was recorded for 30 min and 1 mM CHX was applied in the solution before the roots were pressurized again and Q_v recorded for another 90 min. At the end of this period, root O_2 uptake was determined again for 25 min. The roots were then incubated in aerated distilled water or 1 mM CHX solution for another 90 min and then transferred to distilled water before conducting Q_v measurements for the next 60 min. At the end of Q_v measurements, root O_2 uptake was measured again. For each individual root system, respiration rates were normalized with the initial respiration rate. Since Q_v became stable only after approximately 50 min, the mean Q_v over the first 50 min was used to normalize the Q_v data, for each root system. Six replicates were used for each treatment.

3.2.4. *In vitro* acid phosphatase assay

The *in vitro* acid phosphatase assay was carried out as previously described (Zwiazek and Shay 1988). To obtained a crude enzyme extract, roots were removed from the solution culture and 1g of fresh root tissue was harvested and ground in a mortar with clean sand and 15 ml of cold 50 mM Tris-HCl (pH 7.4), 1% (w/v) polyvinvyl pyrolidone (PVP-40), and 1 mM dithiothreitol (DTT). The homogenate was filtered through 6 layers

of cheesecloth and used immediately. All extraction steps were carried out on ice.

P-nitrophenyl phosphate (P-NPP) was used as a substrate for the acid phosphatase activity measurements. The standard assay solution contained 0.6 mM P-NPP in 50 mM acetate buffer (pH 5.0). Enzyme extract (50 µl) was added to the test tubes containing 2 ml assay solution and 50 µl CHX solution to give a final concentration of 0.25, 0.5, and 1 mM cycloheximide in the reaction mixture. For the control, 50 µl of H_2O was used in place of CHX. The blank test tubes contained homogenization medium in place of enzyme extract. The tubes were placed in a water bath and incubated at 30°C for 25 min. The reaction was stopped with 2 ml of 10% (w/v) Na_2CO_3. The assay was performed using extracts from six root systems. The absorbance of the reaction mixture was measured at 410 nm against the corresponding blank with a UV/Visible spectrophotometer (Ultrospec III, Pharmacea LKB Biotechnology, Uppsala, Sweden). The control value was used to normalize the readings for CHX-treated extracts, for each root extract.

3.2.5. Stomatal conductance (g_s)

In a different experiment, leaf stomatal conductance (g_s) measurements were carried out with a steady-state porometer (LI-1600, LI-COR, Lincoln, NE, U.S.A.) in the same growth chamber where the seedlings were growing. Randomly selected seedlings had their roots placed in 500 ml dark plastic containers filled with distilled water or 1 mM CHX solution (Figure 3.5). A piece of rubber tubing connected each container to an aquarium pump to keep the solution aerated. Measurements were performed on a fully

developed uppermost leaf. Measurements were taken on the same leaf at 0, 30, 60, 90, and 120 min in 6 seedlings per treatment.

3.2.6. Root hydraulic conductivity (L_p)

After stomatal conductance was measured as described above, the roots of those seedlings were excised and their root hydraulic conductivity (L_p) measured using the hydrostatic pressure method (Wan and Zwiazek 2001). Detopped roots were immersed in distilled water inside the pressure chambers as for Q_v measurements. The pressure was gradually increased and maintained at 0.3 MPa for 5 min. Subsequently, the pressure was decreased to 0.15 MPa and then incrementally increased to 0.3, 0.45, 0.60, and 0.75 MPa. At each pressure, the root water flow was allowed to stabilize for 5 min and then Q_v recorded for 15 min, as described above. The water flux density J_v ($m^3\ s^{-1}\ cm^{-3}$) was obtained by dividing Q_v ($m^3\ s^{-1}$) by the root volume (cm^3). Root volume was measured by water displacement method as described by Kamaluddin and Zwiazek (2002). Root hydraulic conductivity (L_p, $m^3\ s^{-1}\ MPa^{-1}\ cm^{-3}$) was calculated as the slope of the regression line of J_v ($m^3\ s^{-1}\ cm^{-3}$) versus applied hydrostatic pressure (MPa).

Root hydraulic conductivity was measured for control and CHX-treated plants after 0, 60, and 120 min of CHX treatment. At each time six different plants were used for each treatment.

3.2.7. Leaf xylem pressure potential (Ψ_w)

For leaf xylem pressure potential (Ψ_w) measurements, seedlings were treated with CHX similarly to those used for stomatal conductance measurements. At times 0, 60 and 120 min after the commencement of CHX treatment, a leaf was detached from each of the 6 control and CHX-treated seedlings and Ψ_w measured with a Scholander pressure chamber (PMS Instruments, Corvallis, OR, U.S.A.), for the uppermost fully developed leaves as described by Wan et al. (1999). At the end of the measurements, roots were excised and used for apoplastic water flow measurements.

3.2.8. Estimation of apoplastic flow ratio

The apoplastic water flux was quantified using a fluorescent tracer dye, trisodium 3-hydroxy-5,8,10-pyrenetrisulphonate (PTS_3) (Hanson et al. 1985, Kamaluddin and Zwiazek 2001). The dye is confined to the apoplast and does not bind to cell walls (Hanson et al. 1985, Moon et al. 1986). Detopped root systems were immersed in 0.02% (w/v) PTS_3 solution inside the pressure chambers and the pressure was increased and maintained constant at 0.3 MPa. Xylem exudates were collected continuous for 0.5-3 h for the analysis of PTS_3 concentration. The PTS_3 concentrations were measured with a Sequoia-Turner 450 fluorometer (Apple Scientific, Chesterland, OH, U.S.A.) using an excitation wavelength of 405 nm and an emission wavelength of 515 nm. Concentrations of PTS_3 in xylem exudates were calculated from standard curve prepared from known PTS_3 concentrations. The proportion of the apoplastic flow was estimated by dividing the

PTS_3 concentration in the xylem sap by the PTS_3 concentration in the root incubating solution. Nine root systems were used for 1 mM CHX treatment and control, six from the Ψ_w measurements plus three additional replicates.

3.2.9. Protein extraction

Microsomal proteins were extracted as described by Henzler et al. (1999) with a few modifications. Four different roots for each of the control, 0.25 mM, 0.5 mM, and 1 mM CHX-treated roots from Q_v measurements were used. Roots were weighed and cut into small pieces (0.5-1 cm length) with scissors. The root segments were vacuum-infiltrated for 30 min in 60 ml ice-cold solution containing 50 mM Hepes buffer, 0.5 M sucrose, 1 mM dithiothreitol (DTT), 5 mM ascorbic acid, 0.6% (w/v) polyvinylpolypyrrolidone (PVPP), and adjusted to pH 7.5 with NaOH.

The material was homogenized, for 45 s with an IKA (Ultra-Turax T-25) homogenizer (IKA Werke Janke & Kunkel GmbH & Co KG, Staufen, Germany). The homogenate was filtered through eight layers of cheesecloth, and the filtrate centrifuged in a Beckman SW28 rotor (Bekman Instruments Inc., Palo Alto, CA, U.S.A.) at 10 000 g for 15 min. The supernatant was further centrifuged at 100 000 g for 30 min to yield a microsomal pellet. The pellet was resuspended in 0.9 ml of 5 mM Mes-Tris buffer (pH 6.5) and stored at -20°C. All extraction steps were carried out on ice.

Microsomal protein content was determined by the bicinchoninic acid (BCA) protein assay (Pierce Chemical, Rokford, Il, USA). Following quantitation, proteins were precipitated from the microsomal samples with 6 volumes of cold ethanol : acetone (1:1

volume), and incubated at -20°C for 1 h. The protein was collected by centrifugation in a centrifuge (Biofuge 13, Heraeus Sepatech GmbH, Osterode, Germany) at 12 000 g for 20 min. Air-dried proteins were stored at -85°C.

3.2.10. Electrophoresis, electroblotting, and immunodetection

Protein samples were dissolved in a sample buffer containing 60 mM Tris-HCl (pH 6.8), 4% (w/v) sodium dodecyl sulfate (SDS), 0.1 M DTT, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, and 0.01% (w/v) bromophenol blue. The samples were heated for 20 min at 56°C and proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), as described by Laemmli (1970), using a 12% acrylamide resolving gel and the Bio-Rad Mini-Protean II apparatus (Bio-Rad Laboratories, Mississauga, ON, Canada). The amount of protein loaded on the gels varied from 160 to 338.9 µg as all samples loaded on a gel represented proteins extracted from the same root fresh weight (0.2 g). Following electrophoresis, gels were stained with Coomassie Brilliant Blue R250 or electroblotted to a nitrocellulose membrane by wet transfer, using the Bio-Rad Mini-Protean II apparatus (Bio-Rad Laboratories, Mississauga, ON, Canada). The nitrocellulose membrane was blocked overnight in 5% (w/v) non-fat milk powder in Tris-buffered saline solution (TBS). The next day, the membrane was incubated for 2 hours with the primary polyclonal antibody (1: 2 000 in 1% milk powder in Tris-buffered saline) raised in chicken against a GST-fusion protein expressing the first 42 NH_2-termini amino acids of the *Arabidopsis thaliana* AthPIP1;1 aquaporin (obtained from Dr. A. Schäffner, Institute for Biochemical Plant Pathology, München, Germany).

The membrane was washed 4x10 min in TTBS (0.05% (v/v) Tween-20 in Tris-bufered saline) and 2x10 min in TBS. The membrane was incubated with alkaline phosphatase-labeled secondary antibody (Sigma, St. Louis, MO, U.S.A.) (1: 5 000 in 1% milk powder in TBS) for 1.5 hours, and washed as described before. To visualize the immunoreactive bands, the alkaline phosphatase assay was carried out using 5-bromo-4-chloro-3-3indolyl phosphate (BCIP) solution (50 mg/ml BCIP in 100% dimethy-formamide DMF) and nitro blue tetrazolium (NBT) solution (50 mg/ml in 70% DMF) in 100 mM Tris buffer pH 9.5 containing 100 mM NaCl, and 5 mM $MgCl_2$.

Protein extraction, electrophoresis, electroblotting and immunodetection was repeated for 16 different samples, representing protein extracted from 4 replicates for each of the control, 0.25 mM, 0.5 mM, and 1.0 mM CHX treatments.

3.2.11. Statistical analysis

Statistical analysis for Q_v, root respiration, stomatal conductance, and Ψw were performed using the Mixed Procedure of SAS (version 8.1, SAS Institute, Cary, NC). The measured characteristics were analyzed as a repeated measures analysis of variance. The fixed effects in the model were the treatments (different CHX solutions applied in the root incubating solution). For completion, experiments had to be carried out in consecutive days. In all statistical models, a Kenward-Roger option was used to calculate denominator degrees of freedom. For each model a Variance-Covariance Matrix was chosen based on the Schwarz's Baysian Criterion (SAS 2000). Least Square Means and standard errors were calculated for treatments, days, time and treatment*time interaction

effects and the Least Square Means separated using a pdiff option.The statistical model was:

$$Y_{ijkl} = \mu + T_i + D_j + P_k + TP_{ik} + e_{ijkl}$$

Where Y_{ijkl} = value of individual observation

μ = overall mean

T_i = effect of i^{th} treatment (i = the level of treatments)

D_j = effect of j^{th} day (j = the level of day)

P_k = effect of k^{th} period (k = the level of period)

TP_{ik} = interaction effect of i^{th} treatment with k^{th} period

e_{ijkl} = random error (l = 1, 2,….., number of replicates)

The phosphatase assay, root hydraulic conductivity (L_p), and apoplastic water flow data were analyzed using GLM Procedure of SAS (version 8.1, SAS institute, Cary, NC). The statistical model was:

$$Y_{ijk} = \mu + T_i + D_j + TD_{ij} + e_{ijk}$$

Where Y_{ijkl} = value of individual observation

μ = overall mean

T_i = effect of i^{th} treatment (i = the level of treatments)

D_j = effect of j^{th} day, or group (j = the level of day)

TD_{ij} = interaction effect of i^{th} treatment with j^{th} day or group

e_{ijk} = random error (l = 1, 2,….., number of replicates)

Least Square Means were separated using a pdiff option. All differences were tested at the $\alpha \leq 0.05$ level.

3.3. Results

3.3.1. Effects on Q_v

Three different concentrations of CHX were tested for their effects on Q_v (Figure 3.1-A). Following CHX application, Q_v values were similar to those of control for about 20-25 min, at which point Q_v started to decrease. After 30 min from the initiation of treatments, the decrease in Q_v became statistically significant for the 1 mM CHX treatment (11% decrease, $P = 0.0316$). For 0.25 and 0.5 mM CHX treatments, Q_v became statistically different from the control (10% decrease, $P = 0.0445$ and 13% decrease, $P = 0.0111$, respectively) after 35 min from CHX application (Figure 3.1-A). After 35 min Q_v continued to decrease steadily for all CHX treatments. After 90 min from the CHX application, Q_v values declined by 41%, 51%, and 58% compared with control in 0.25 mM, 0.5 mM, and 1 mM CHX treatments, respectively ($P < 0.0001$) (Figure 3.1-A).

3.3.2. Protein expression

Western blots of the root microsomal proteins were probed with a polyclonal antibody raised against 42 NH_2-terminal amino acids residues from *Arabidopsis thaliana* AthPIP1;1 aquaporin. The NH_2-terminal of plant PIP1 water channel proteins is conserved and the antibodies have been demonstrated to immunoreact with membrane protein preparations from several plant species (Schäffner 1998, Henzler et al. 1999). In the present study, the antibodies recognized a single protein band in CHX-treated and

control plants with an apparent molecular weight of approximately 32 kDa (Figure 3.1-B). The immunoreactive band showed a decrease in the intensity of immunostaining with increasing CHX concentrations (Figure 3.1-B). In the 1 mM CHX treatment, a lower molecular weight immunoreactive band was detected (approximately 23 kDa). The 23 kDa band was observed with a lower intensity in the lower CHX concentration treatments (not shown in Figure 3.1-B). The results were similar for all samples in 4 repeated extractions.

3.3.3. Effects on root respiration

In a repeated experiment, root oxygen uptake was determined for 1 mM CHX and related to Q_v (Figure 3.2-A, B). Root oxygen uptake rates decreased over time in control and CHX-treated roots. At time 90 min, the O_2 uptake values were slightly, but significantly ($P = 0.0148$) higher in CHX-treated compared to control roots (Figure 3.2-B). At the same time, there was a significant decrease in Q_v values of CHX-treated roots ($P < 0.0001$) (Figure 3.2-A). Following incubation, Q_v of control roots gradually increased for about 45-50 min, while Q_v of CHX-treated roots was maintained at a constant low rate. There was no difference in root O_2 uptake at time 265 min between the control and CHX-treated roots (Figure 3.2-B). However, Q_v was lower by 61% ($P < 0.0001$) in CHX-treated roots compared with control at time 265 min.

3.3.4. *In vitro* acid phosphatase activity

Cycloheximide had a little effect on the *in vitro* activity of acid phosphatase (Figure 3.3). Only 1 mM CHX showed a 7% (P = 0.0024) decrease in the activity of acid phosphatase, when compared with control extracts.

3.3.5. Seedling water relations

3.3.5.1. Stomatal conductance (g_s) and root hydraulic conductivity (L_p)

In a repeated experiment, seedlings were randomly assigned to control and 1 mM CHX treatments and g_s monitored for two hours. During the first hour, leaf stomatal conductance (g_s) was similar in both control and CHX-treated plants (Figure 3.4). After 60 min from the treatment initiation, g_s of CHX-treated seedlings started decreasing. However, the decrease became significant only after another 60 min (P = 0.0015).

The above effect on g_s was accompanied by visible changes in the appearance of seedlings. One hour after the commencement of CHX treatment, leaves of the CHX-treated seedlings started to droop and after 2 h, some of the seedlings were wilted (Figure 3.5).

Pressure-flux responses for control and CHX-treated seedlings for 1 and 2 h showed a linear relationship from 0.15 to 0.75 MPa (Figure 3.6-A). The mean slopes of the linear relationships between J_v and pressure were used to calculate root hydraulic conductivity (L_p), for each root system. In seedlings treated with CHX for 1 and 2 h, L_p

showed 85% and 87% decline ($P \leq 0.05$), respectively, compared with control (Figure 3.6-B).

3.3.5.2. Leaf xylem pressure potential (Ψ_w) and apoplastic water flow

Following CHX application, the leaf xylem pressure potential (Ψ_w) started to decline (Figure 3.7-A). However, the decrease became significant only after 2 h from the treatment initiation ($P = 0.0007$).

Apoplastic water flow determinations were performed for the same roots for which the leaf water potential was measured. For the control roots, PTS_3 concentration in the xylem sap was about 1.7% of the PTS_3 concentration present in the root bathing solution (Figure 3.7-B). The decrease in Ψ_w after two hours exposure to CHX was accompanied by an almost three fold increase ($P = 0.0121$) in the PTS_3 concentration of the xylem sap (Figure 3.7-B), meaning an increase in the apoplastic water transport.

3.4. Discussion

The present study demonstrated that protein-mediated water transport is a major factor controlling plant water relations in aspen (*Populus tremuloides* Michx.) seedlings. The quick response, observed within 90 min, to CHX treatment also suggests that plants may use transcriptional or translational regulation of root aquaporins for quick adjustment of hydraulic conductivity. Recent studies using anti-sense plants have shown the

importance of aquaporins for the whole plant water transport (Kaldenhoff et al. 1998, Siefritz et al. 2002).

In the present study, the inhibition of Q_v by CHX showed a different pattern than the $HgCl_2$ inhibition reported in previous studies (Wan and Zwiazek 1999, Kammaluddin and Zwiazek 2002). While $HgCl_2$ directly blocks the aquaporin pore (Tyreman et al. 1999), an inhibition of protein synthesis may take longer time to induce physiological responses. However, after 20-30 min following CHX application, Q_v started decreasing steadily in all CHX concentrations used. The steady-state root water flow was reduced by 41%, 51%, and 58% in 0.25, 0.5, and 1 mM CHX, respectively, after 90 min of treatment. Water flow across the plasma membrane is facilitated largely by water channel proteins (Kammerloher et al. 1994, Maurel 1997, Tyreman et al. 1999). Our previous studies suggested that in aspen roots, these proteins play a dominant role in regulating root water flow (Wan and Zwiazek 1999, Kamaluddin and Zwiazek 2002). The inhibition of Q_v in CHX-treated plants was likely mediated by aquaporins. In wheat (*Triticum aestivum* L.), 0.25 and 1 mM CHX reduced the incorporation of ^{35}S into microsomal proteins by 68% and 72%, respectively, with 21% and 40% reductions into total proteins (Zhang et al. 1995), suggesting that the initial target of CHX are membrane proteins. This could explain a rapid and profound effect of CHX on Q_v in the present study. As a protein inhibitor, CHX may also affect regulatory proteins that are involved in regulating the aquaporin activity. However, in the present study we demonstrated that the reduction of Q_v in CHX-treated roots was accompanied by a reduction in expression of a 32 kDa immunoreacted protein. The protein immunoreacted with a polyclonal antibody raised against 42 NH_2-terminal amino acids residues from *Arabidopsis thaliana* AthPIP1;1

aquaporin. Aquaporins are highly conserved membrane proteins with molecular weights ranging from 26 to 34 kDa (Tyreman et al. 2002). Therefore, in the present study, the recognized protein was likely related to the PIP1 aquaporin. It has been shown that transcriptional regulation of aquaporin genes can operate in less than one hour (Kawasaki et al. 2001) but, as pointed out by Javot and Maurel (2002), there may also be a combination of other regulatory mechanisms. Since 1 mM CHX reduced Q_v by 58% within 90 min, the turnover rate of proteins that control root water uptake appears to be rapid and of major importance for root water uptake. The present study provides direct evidence of the magnitude of protein-mediated water transport in plant roots.

Immunostaining also revealed the appearance of a 23 kDa protein on roots treated with 1 mM CHX. Barone et al. (1997) showed that Hg^{2+} induced the formation of a dose-dependent proteolitic fragment of 22-kDa from the plasma membrane aquaporins from *Beta vulgaris*. In the present study, the 23-kDa band could be a result of proteolysis. However, since CHX inhibits peptide initiation and extension (O'Brig et al. 1971), it is also likely that the band represents an incomplete immunoreactive product of aquaporin synthesis. Since, the 23 kDa protein did not appear in the control lane, it is unlikely that the fragment resulted during protein extraction, as a result of protein degradation.

Root water uptake and the activity of root aquaporins are often affected by root respiration (Tyreman et al. 1999, Zhang and Tyreman 1999, Kamaluddin and Zwiazek, 2001), possible due to links with phosphorylation and dephosphorylation processes (Johansson et al. 1996). However, in the present study, O_2 uptake rates were similar in CHX-treated and control roots. A decrease in respiration was observed in pressurized roots of CHX-treated and control, and was probably due to the filling of the previously

aerated root air spaces with water (Markhart and Smith 1990). This decrease was probably partly responsible for the decline in Q_v in all roots. Prolonged exposure to CHX may also severely disrupt overall metabolism (Zhang et al. 1995) and result in a depolarization of the plasma membrane (Pakhomova and Pakhnov 1992), as well as a decrease in phosphate uptake (Clarkson et al. 1992). However, in the present study, there was no difference between O_2 uptake rates of control and CHX-treated roots after 265 min of treatments. At the same time, there was a large decrease in Q_v of CHX-treated roots. This suggests that the inhibition of Q_v by CHX was not due to general metabolic depression. Although we cannot exclude the possibility that CHX indirectly affected phosphorylation and dephosphorylation events, we tested its effects on the *in vitro* acid phosphatase activity using control root extracts. Of the tested concentrations, only the highest, 1 mM CHX had a slight inhibitory effect. Therefore, CHX probably did not affect phosphorylation and dephosphorylation processes in intact roots. Therefore, it appears that most of the root water permeability in aspen seedlings is mediated by proteins.

Previous studies have shown that mercury-sensitive processes of water transport in aspen roots affect the plant water balance through their effects on L_p (Wan and Zwiazek 1999). However, in addition to blocking water channels, mercury is a cytotoxin (Barrowclough et al. 2000), and affects cell metabolism and oxygen uptake (Kamaluddin and Zwiazek 2001). By blocking protein synthesis (O'Brig et al. 1971), cycloheximide is a useful tool in the initial assessment of the protein-mediated water transport. Cycloheximide has been successfully used to inhibit aquaporin synthesis in animal cells (Katsura et al. 1996). Complete inhibition of AQP-2 biosynthesis by 0.03 mM

cycloheximide in transfected LLC-PK_1 epithelial cells was verified by immunoprecipitation (Katsura et al. 1996). Incubation with 2 mM cycloheximide for 7 or 12 hours had been able to inhibit osmotic water permeability of motor cell protoplast in *Samanea samanea* (Moshelion et al. 2002).

Cycloheximide was applied to roots of intact plants to investigate the effects of CHX on plant water relations. In the present study, stomatal conductance was reduced within 2 h following the application of 1 mM CHX to roots. There was also a drastic reduction in the root water flux density (J_v) within 1 h. The L_p values in CHX-treated roots declined by 85% and 87% of the control values within 1 and 2 h, respectively. Similarly to g_s, leaf xylem pressure potential (Ψ_w) was reduced within 2 h. It is possible that hydraulic signals originating in the roots were responsible for the stomatal closure. However, the involvement of chemical signals cannot be discounted. As suggested by Else et al. (1995) for flooded tomato plants, a decrease in leaf water potential is not sufficient to markedly influence stomatal conductance. It is known that environmental stresses such as drought and low temperature can trigger ABA synthesis, which, in turn, can induce stomatal closure (Zhang et al. 1987, Lee et al. 1993). However, in intact plants, CHX has been shown to maintain stomatal opening (Thimann and Tan 1988, Li and Walton 1990) by abolishing ABA synthesis (Popova et al. 2000). As a consequence, stomatal closure could be brought about by the intraleaf ABA redistribution from ABA stores within leaf symplast (located in mesophyll and epidermis) (Popova et al. 2000).

In the present study, the apoplastic water flow was also quantified following 2 h of CHX treatment in intact seedlings. The fluorescent dye PTS_3 is restricted to apoplast and does not penetrate the cells (Hanson et al. 1985, Moon et al. 1986). The concentration of

PTS_3 in the xylem exudates of control seedlings was 1.7% of the PTS_3 concentration present in the root medium, suggesting that the cell-to-cell water flow was the predominant pathway in aspen roots. Other studies using fluorescent tracer dyes also suggest that only a small fraction of water is transported through the apoplastic pathway (Hanson et al. 1985, Skiner and Radin 1994, Wan and Zwiazek 1999, Kamaluddin and Zwiazek 2001). However, Zimmermann and Steudle (1998) pointed out that the PTS_3 may underestimate the apoplastic water flow. The apoplastic flow of PTS_3 appears to be limited by the presence of endodermis (Zimmermann and Steudle 1998). However, in the present study, CHX-treated roots showed a several-fold increase in the PTS_3 concentration of the xylem exudates. This increase in the apoplastic flow indicates that in CHX-treated roots there was an increase in the apoplastic water transport. These results confirm that CHX affected the cell-to-cell pathway in roots.

3.5. Conclusions

In conclusion, our study provided direct evidence that protein synthesis plays a major role in plant water uptake. Contrary to the initial hypothesis, the rate of membrane protein turnover appears to be fast and could be used for rapid regulation of root water transport. At the whole seedling level, the inhibition of root hydraulic conductivity triggered decreases in leaf water potential and stomatal conductance. In the present study, the contribution of plasmodesmata to the overall water transport was not assessed and the effect of CHX on symplastic water transport cannot be discounted.

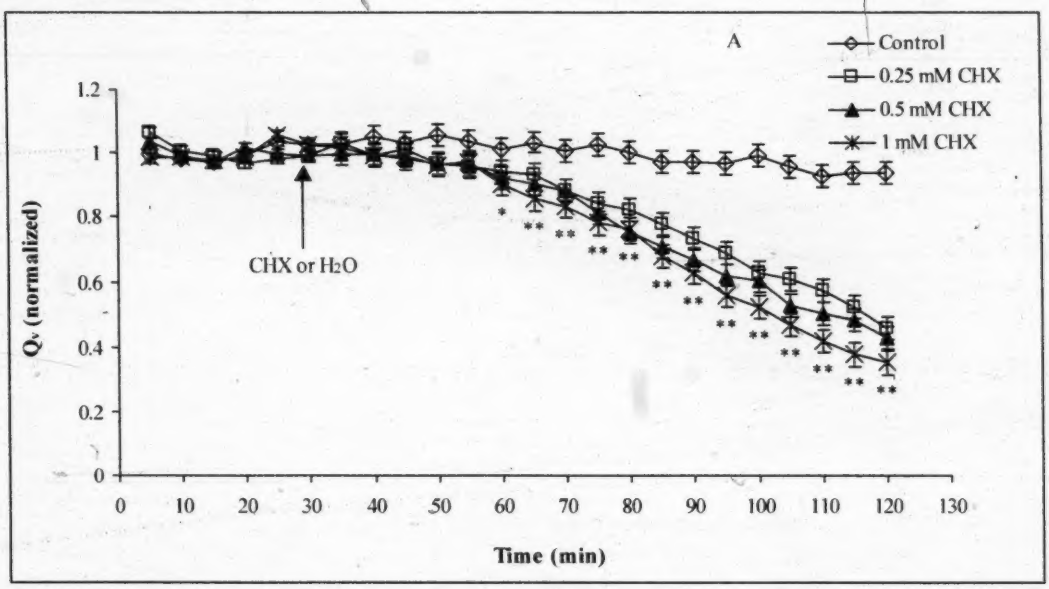


Figure 3.1 (A) The time course of the steady-state root water flow (Q_v) in response to different cycloheximide (CHX) concentrations. Mean Q_v over the initial 30 min was used to normalize the data. Data points represent adjusted least square means ± SE (n=6). The arrow mark indicates the time of CHX or water application. (* Q_v for 1 mM CHX significantly different from the control; ** Q_v of all CHX treatments significantly different from control) ($P \leq 0.05$). (B) Western blots of the microsomal proteins extracted from the same roots used in (A). First lane represents protein standards. Lanes 2-5 represent microsomal protein from roots treated with different CHX concentrations. Microsomal proteins were separated by SDS-PAGE. Protein bands immunoreactive with AthPIP1;1 antibody were visualized by the alkaline phosphatase immunoassay.

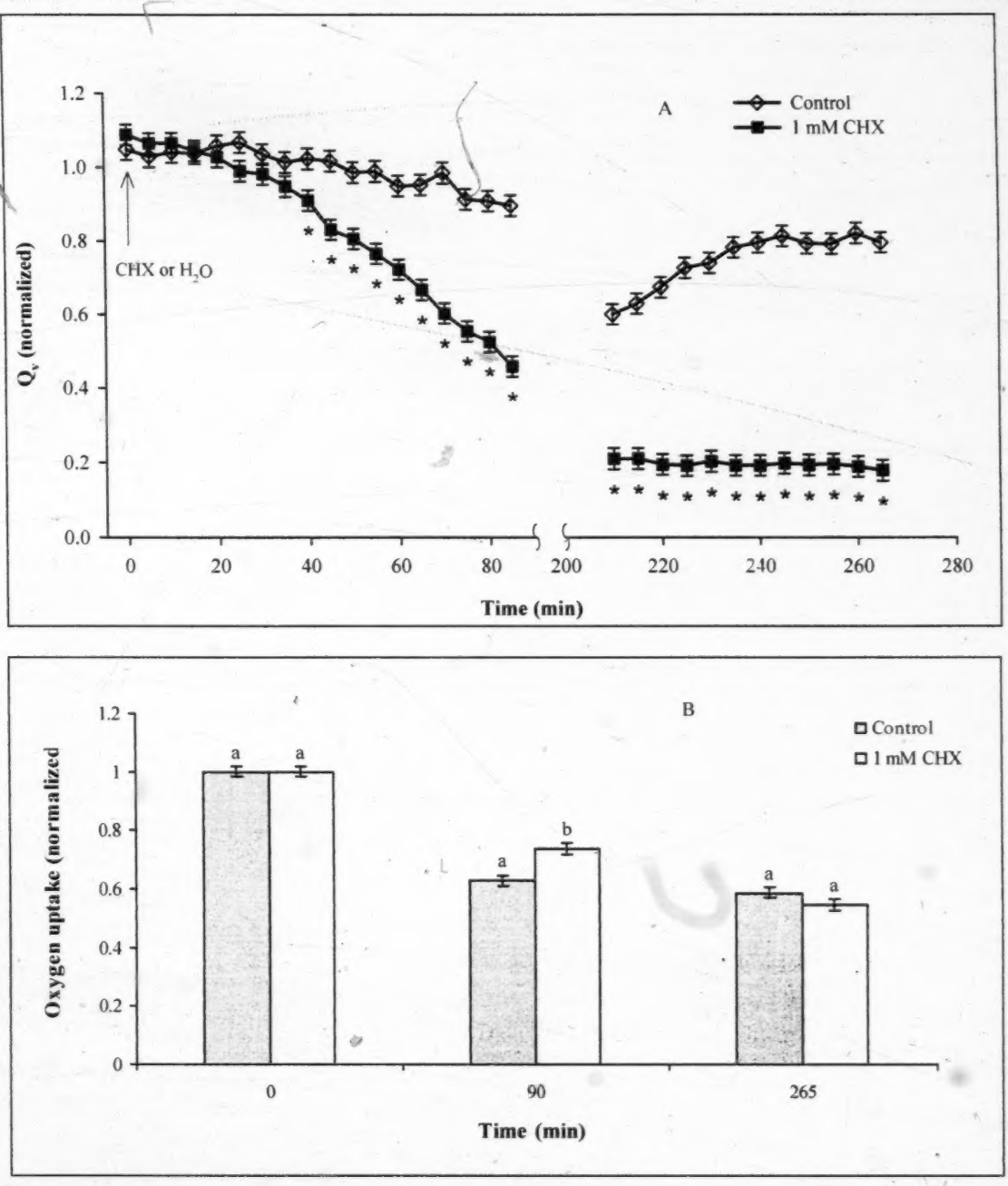


Figure 3.2. Time course of Q_v (A) and root respiration (B) for the same roots following application and incubation in 1 mM cycloheximide (CHX) or distilled water (control). Least square means ± SE (n=6) are shown. (A) Arrow mark indicates the time of CHX or water application. (* significant differences at P≤0.05 level). (B) The O_2 uptake rates obtained at time 0 were used to normalize the respiration rates following treatment. Bars in the same group followed by different letters are significantly different (P≤0.05).

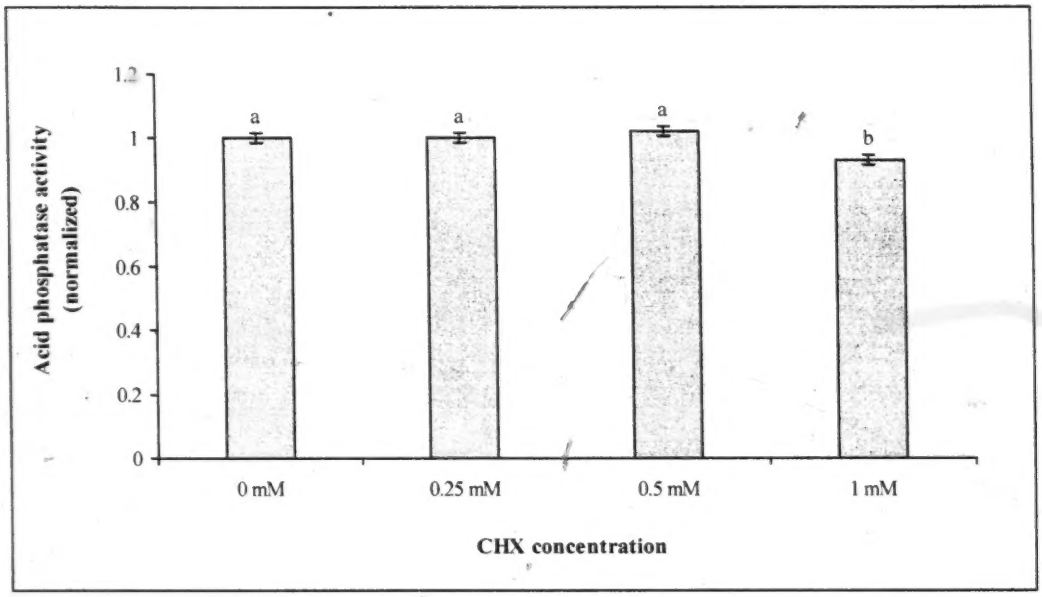


Figure 3.3 The *in vitro* assay of the cycloheximide (CHX) effect on acid phosphatase activity. Control values were used to normalize the data. Least square means ± SE are presented (n=6). Different letters indicate significant differences (P≤0.05).

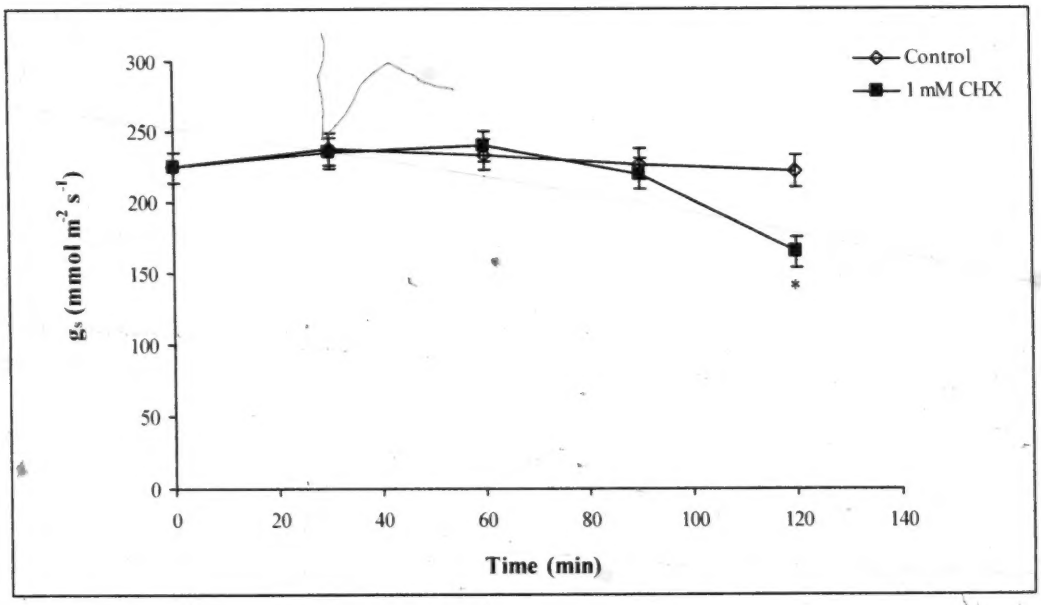


Figure 3.4 Stomatal conductance (g_s) in seedlings treated with 1 mM CHX solution applied to roots. Data points are least squares means ± SE (n=6). * represents g_s significantly different from the control ($P \leq 0.05$).

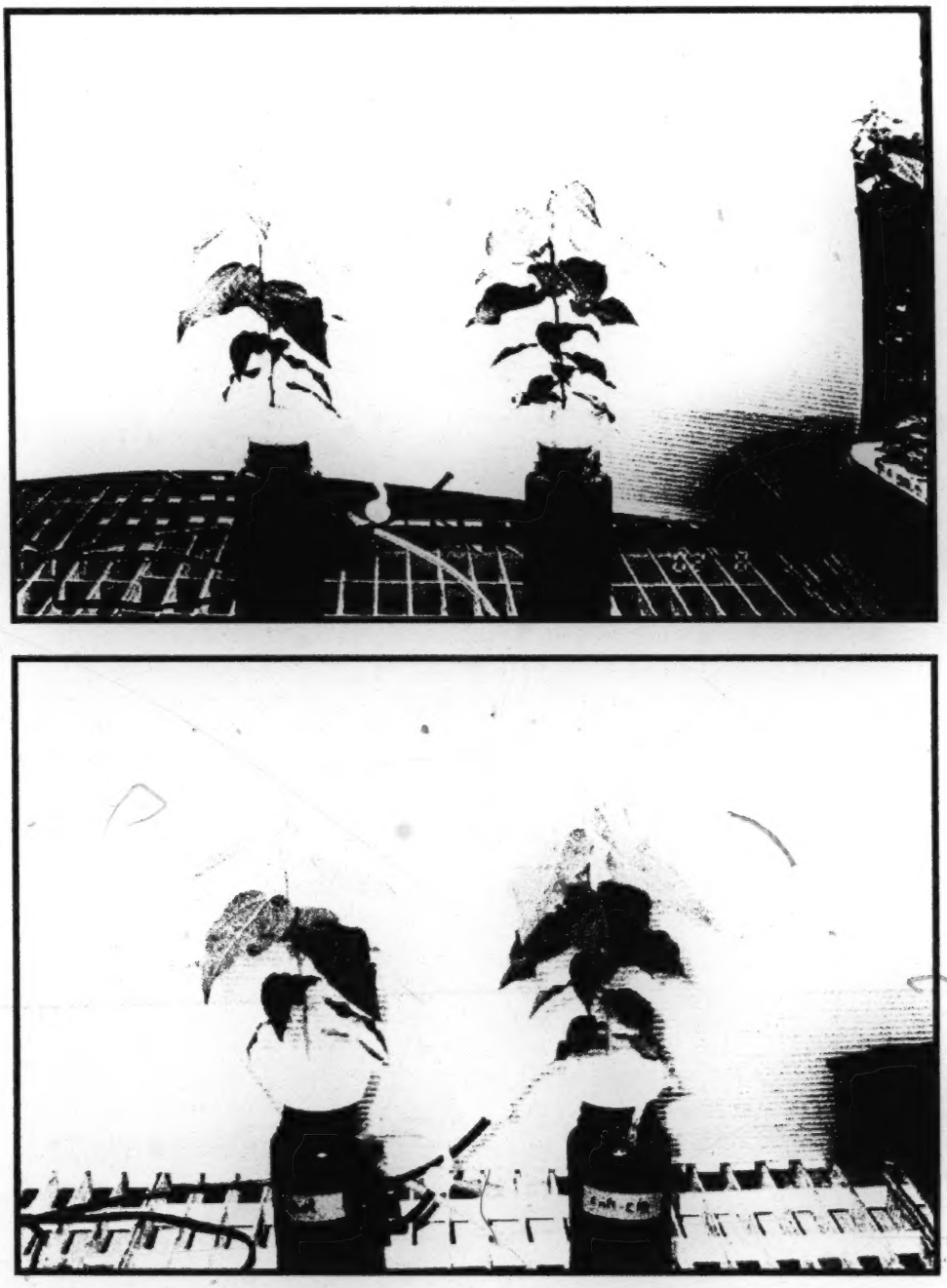


Figure 3.5 Experimental set-up for stomatal conductance measurements. Control (left) and 1 mM CHX-treated plants (right) are presented, at the beginning (upper picture) and after 2 h (lower picture) of treatment application. The leaves of CHX-treated plants (right) started drooping after 2-h treatment.

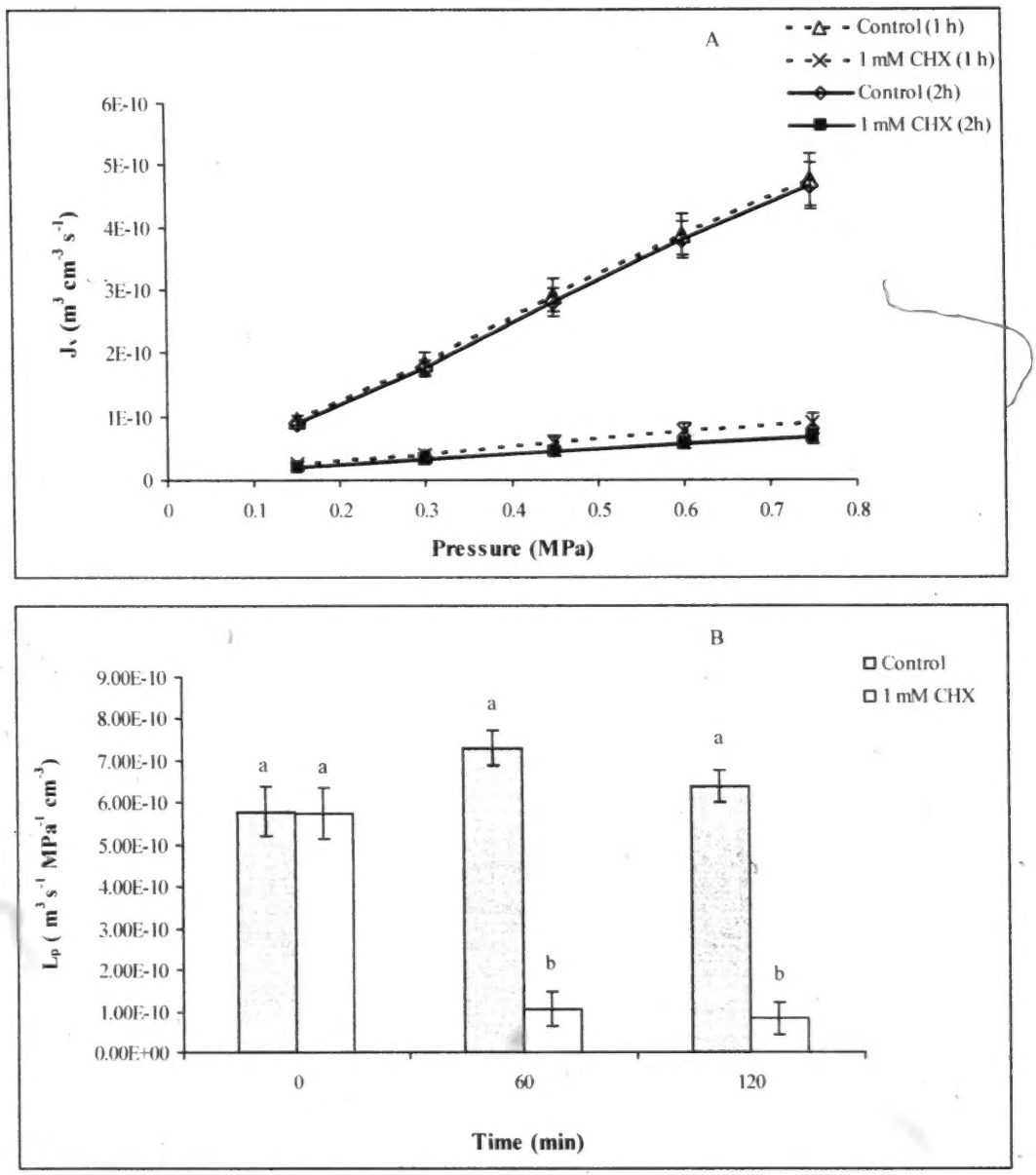


Figure 3.6 Effect of cycloheximide (CHX) on (A) water flux density (J_v) and (B) root hydraulic conductivity (L_p). (A) Means ± SE (n=12) are represented. (B) Least square means ± SE (n=6) are presented. Bars in the same group with different letters are significantly different ($P \leq 0.05$).

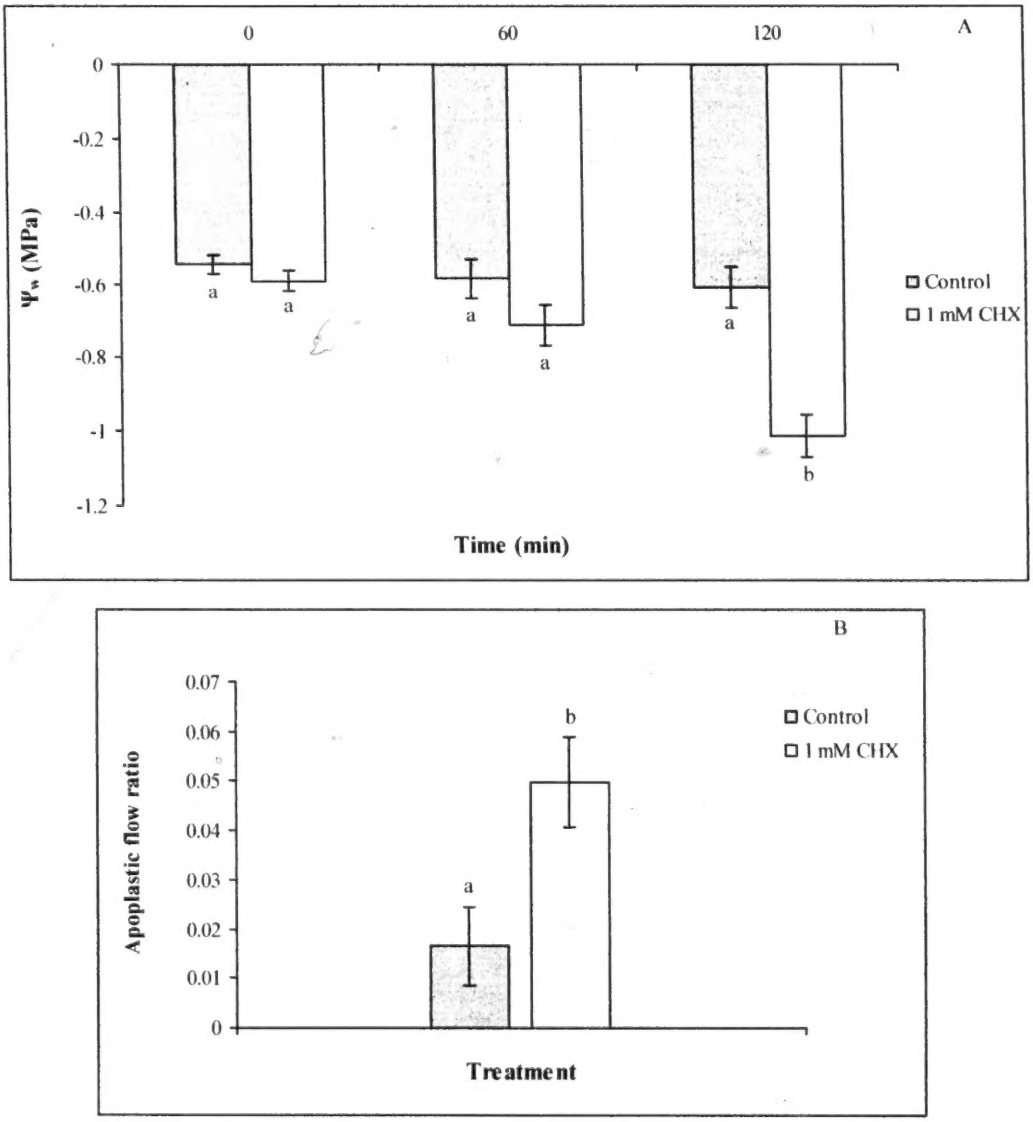


Figure 3.7 (A) Leaf xylem pressure potential (Ψ_w) in control and cycloheximide (CHX)-treated seedlings. (B) Apoplastic flow ratio calculated as the ratio between PTS_3 concentration in the xylem exudates and PTS_3 concentration in the root bathing solution after 2 h CHX treatment. Bars represent least square means ± SE (n=6). Bars with different letters in the same group are significantly different ($P \leq 0.05$).

References

Barone, L.M., Shih, C., and Wasserman, B.P. 1997. Mercury-induced conformational changes and identification of conserved surface loops in plasma membrane aquaporins from higher plants. J. Biol. Chem. 272(49): 30672-30677.

Barrowclough, D.E., Peterson, C.A., and Steudle, E. 2000. Radial conductivity along developing onion roots. J. Exp. Bot. 344: 547-557.

Carvajal, M., Cerdá, A., and Martinez, V. 2000. Does calcium ameliorate the negative effect of NaCl on melon root water transport by regulating aquaporin activity? New Phytol. 145: 439-447.

Carvajal, M., Martinez, V., and Alcaraz, C.F. 1999. Physiological function of water channels as affected by salinity in roots of paprika pepper. Physiol. Plant. 105: 95-101.

Chaumont, F., Barrieu, F., Jung, R., and Chrispeels, M.J. 2000. Plasma membrane intrinsic proteins from maize cluster in two sequence subgroups with differential aquaporin activity. Plant Physiol. 122: 1025-1034.

Clarkson, D.T., Carvajal, M., Henzler, T., Waterhouse, R.N., Smyth, A.J., Cooke, D.T., and Steudle, E. 2000. Root hydraulic conductance: diurnal aquaporin expression and the effects of nutrient stress. J. Exp. Bot. 51(342): 61-70.

Clarkson, D.T., Hawkesford, M.J., Davidian, J.-C., Grignon, C. 1992. Contrasting responses of sulphate and phosphate transport in barley (*Hordeum vulgare* L.) roots to protein -modifying reagents and inhibition of protein synthesis. Planta 187: 306-314.

Else, M.A., Davies, W.J., Malone, M., and Jackson, M.B. 1995. A negative hydraulic message from oxygen-deficient roots of tomato plants? Plant Physiol. 109: 1017-1024.

Fiscus, E.L. 1975. The interaction between osmotic and pressure-induced water flow in plant roots. Plant. Physiol. 55: 917-922.

Fotidias, D., Jenö, P., Mini, T., Wirtz, S., Müller, S.A., Fraysse, L., Kjelbom, P., and Engel, A. 2001. Structural characterization of two aquaporins isolated from native spinach leaf plasma membrane. J. Biol. Chem. 276 (3): 1707-1714.

Hanson, P.J., Sucoff, E.I., and Markhart III, A.H. 1985. Quantifying apoplastic flux through red pine root systems using trisodium, 3-hydroxy-5,8,10-pyrenetrisulfonate. Plant Physiol. 77: 21-24.

Harvengt, P., Vlerick, A., Fuks, B., Wattiez, R., Ruysschaert, J.M., and Homble, F. 2000. Lentil seed aquaporins form a hetero-oligomer which is phosphorylated by a Mg^{2+}-dependent and Ca^{2+}-regulated kinase. Biochem. J. 352: 183-190.

Henzler, T., Waterhouse, R.N., Smyth, A.J., Carvajal, M., Cooke, D.T., Schäffner, A.R., Steudle, E., and Clarkson, D.T. 1999. Diurnal variations in hydraulic conductivity and root pressure can be correlated with the expression of putative aquaporins in the root of *Lotus japonicus*. Planta 210: 50-60.

Javot, H., and Maurel, C. 2002. The role of aquaporins in root water uptake. Ann. Bot. 90: 301-313.

Johansson, I., Karlsson, M., Shukla, V.K., Chrispeels, M.J., Larsson, C., and Kjellbom, P. 1998. Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. Plant Cell 10: 451-460.

Johansson, I., Larsson, C., Ek, B., and Kjellbom, P. 1996. The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^{2+} and apoplastic water potential. The Plant Cell 8: 1181-1191.

Kaldenhoff, R., Grote, K., Zhu, J.J., and Zimmermann, U. 1998. Significance of plasmalemma aquaporins for water transport in *Arabidopsis thaliana*. Plant J. 14(1): 121-128.

Kamaluddin, M., and Zwiazek, J.J. 2001. Metabolic inhibition of root water flow in red-osier dogwood (*Cornus stolonifera*) seedlings. J. Exp. Bot. 52 (357): 739-745.

Kamaluddin, M., and Zwiazek, J.J. 2002. Ethylene enhances water transport in hypoxic aspen. Plant Physiol. 128: 1-8.

Kammerloher, W., Fischer, U., Piechottka, G.P., and Schäffner, A.R. 1994. Water channels in the plant plasma membrane cloned by immunoselection from a mammalian expression system. Plant J. 6: 187-199.

Katsura, T., Ausiello, D.A., and Brown, D. 1996. Direct demonstration of aquaporin-2 water channel recycling in stably transfected LLC-PK1 epithelial cells. Am. J. Physiol. 270(3 part 2): F548-F553.

Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D., and Bohnert, H.J. 2001. Gene espression profiles during the initial phase of salt stress in rice. The Plant Cell 13: 889-905.

Kirch, H.H., Vera-Estrella, R., Golldack, D., Quigley, F., Michalowski, C.B., Barkla, B.J., and Bohnert, H.J. 2000. Expression of water channel proteins in *Messembryanthemum crystallinum*. Plant Physiol. 123: 111-124.

Lee, T.-M., Lur, H.-S., and Chu, C. 1993. Role of abscisic acid in chilling tolerance of rice (*Oryza sativa* L.) seedlings. I. Endogenous abscisic acid levels. Plant Cell Environ. 16: 481-490.

Li, Y., and Walton, D.C. 1990. Effects of cycloheximide on abscisic acid biosynthesis and stomatal aperture in bean leaves. Plant Physiol. 93: 128-130.

Lu, Z., and Neumann, P.M. 1999. Water stress inhibits hydraulic conductance and leaf growth in rice seedlings but not the transport via mercury-sensitive water channels in the root. Plant Physiol. 120: 143-151.

Maggio, A., and Joly, R.J. 1995. Effects of mercuric chloride on the conductivity of tomato root system. Plant Physiol. 109: 331-335.

Markhart, A.H., and Smit, B. 1990. Measurement of root hydraulic conductance. HortSci. 25: 282-287.

Martinez-Ballesta, M.C., Martinez, V., and Carvajal, M. 2000. Regulation of water channel activity in whole roots and in protoplasts from roots of melon plants grown under saline conditions. Aust. J. Plant Physiol. 27: 685-691.

Maurel, C. 1997. Aquporins and water permeability of plant membranes. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 399-420.

Maurel, C., Kado, R.T., Guern, J., and Chrispeels, M.J. 1995. Phosphorylation regulates the water channel activity of the seed-specific aquaporin α-TIP. The EMBO J. 14(13): 3028-3035.

Moon, G.J., Clough, B.F., Peterson, C.A., and Allaway, W.G. 1986. Apoplastic and symplastic pathways in *Avicennia marina* (Forsk.) Vierh. Roots revealed by fluorescent tracer dyes. Aust. J. Plant Physiol. 13: 637-648.

Moshelion, M., Becker, D., Biela, A., Uehlein, N., Hedrich, R., Otto, B., Levi, H., Moran, N., and Kaldenhoff, R. 2002. Plasma membrane aquaporins in the motor cells of *Samanea saman*: diurnal and circadian regulation. Plant Cell 14: 727-739.

Nobel, P.S., Schulte, P.J., and North, G.B. 1990. Water influx characteristics and hydraulic conductivity for roots of *Agave deserti* Engelm. J. Exp. Bot. 225: 409-415.

O'Brig, T.G., Culp, W.J., McKeenan, W.L., and Boyd, H. 1971. The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis in reticulocyte ribosomes. J. Biol. Chem. 246(1): 174-181.

Pakhomova, V.M., and Pakhomov, D.V. 1992. Problem on existence of protein, hyperpolarizing wheat root cells under the stress. Fiziol. Biokhim, Kul'Tur. Ras. 24(2): 159-163.

Passioura, J.B. 1988. Water transport in and to roots. Ann. Rev. Plant. Physiol. Plant Mol. Biol. 34: 245-265.

Popova, L.P., Outlaw, W.H. Jr., Aghoram, K., and Hite, D.R.C. 2000. Abscisic acid - an intraleaf water-stress signal. Physiol. Plant. 108: 376-381.

SAS 2000. SAS/STAT User's Guide (Version 8). SAS Inst. Inc., Cary, NC.

Schäffner, A.R. 1998. Aquaporin function, structure, and expression: are there more surprises to surface in water relation? Planta 204(2): 131-139.

Siefritz, F., Tyree, M.T., Lovisolo, C., Schubert, A., and Kaldenhoff, R. 2002. PIP1 plasma membrane aquaporins in tobacco: from cellular effects to function in plants. Plant Cell 14: 869-876.

Skinner, R.H., and Radin, J.W. 1994. The effect of phosphorus nutrition on water flow through the apoplastic bypass in cotton roots. J. Exp. Bot. 45(273): 423-428.

Steudle, E. 2000. Water uptake by the plant roots: an integration of views. Plant Soil 226: 45-56.

Steudle, E., and Frensch, J. 1996. Water transport in plants: role of the apoplast. Plant Soil 187: 67-79.

Steudle, E., and Peterson, C.A. 1998. How does water get through roots? J. Exp. Bot. 49(322): 775-778.

Suga, S., Imagawa, S., and Maeshima, M. 2001. Specificity of the accumulation of mRNAs and proteins of the plasma membrane and tonoplast aquaporins in radish organs. Planta 212: 294-304.

Tazawa, M., Ohkuma, E., Shibasaka, M., and Nakashima, S. 1997. Mercurial-sensitive water transport in barley roots. J. Plant Res. 110: 435-442.

Thimann, K.V., and Tan, Z.-Y. 1988. The dependence of stomatal closure on protein synthesis. Plant Physiol. 86: 341-343.

Tyreman, S.D., Bohnert, H.J., Maurel, C., Steudle, E., and Smith, J.A.C. 1999. Plant aquaporins: their molecular biology, biophysics and significance for plant water relations. J. Exp. Bot. 50: 1055-1071.

Tyreman, S.D., Niemietz, C.M., and Bramley, H. 2002. Plant aquaporins: multifunctional water and solute chanls with expanding roles. Plant Cell Environ. 25: 173-194.

Wan, X., Landhäusser, S.M., Zwiazek, J.J., and Lieffers, V.J. 1999. Root water flow and growth of aspen (*Populus tremuloides*) at low root temperatures. Tree Physiol. 19: 879-884.

Wan, X., and Zwiazek, J.J. 1999. Mercuric chloride effects on root water transport in aspen seedlings. Plant Physiol. 121: 939-946.

Wan, X., and Zwiazek, J.J. 2001. Root water flow and leaf stomatal conductance in aspen (*Populus tremuloides*) seedlings treated with abscisic acid. Planta 213: 741-747.

Yamamoto, N., Sobue, K., Miyachi, T., Inagaki, M., Miura, Y., Katsuya, H., and Asai, K. 2001. Differential regulation of aquaporin expression in astrocytes by protein kinase C. Molec. Brain Res. 95: 110-116.

Zhang, G., Archambault, D.J., Slaski, J.J., and Taylor, G.J. 1995. Effects of protein synthesis inhibitor on uptake of aluminium in aluminium-resistant ans aluminium-sensitive cultivars of wheat. J. Plant Physiol. 147: 457-462.

Zhang, J., Schurr, U., and Davies, W.J. 1987. Control of stomatal behaviour by abscisic acid which apparently originates in the roots. J. Exp. Bot. 38(192): 1174-1181.

Zhang, W.H., and Tyreman, S.D. 1999. Inhibition of water channels by $HgCl_2$ in intact wheat root cells. Plant Physiol. 120: 849-857.

Zimmermann, H.M., Hartmann, K., Schreiber, L., and Steudle, E. 2000. Chemical composition of apoplastic barriers in relation to radial hydraulic conductivity of corn roots (*Zea mays* L.). Planta 210: 302-311.

Zimmermann, H.M., and Steudle, E. 1998. Apoplastic transport across young maize roots: effects of the exodermis. Planta 206: 7-19.

Zwiazek, J.J., and Shay, J.M. 1988. Sodium fluoride induced metabolic changes in jack pine seedlings. II. Effects on growth, acid phosphatase, cytokinins, and pools of soluble proteins, amino acids, and organic acids. Can. J. For. Res. 18: 1311-1317.

Chapter 4. Synthesis

4.1. General discussion and conclusions

The general objective of this study was to assess the involvement of phosphorylation and dephosphorylation and protein synthesis events in the root water uptake processes of woody plants. The importance of these events was to be evaluated by the time course of inhibition or stimulation of water transport in the roots of aspen seedlings. Aspen was chosen for this study because of its ecological and commercial importance and because it has been previously used as a model woody plant for studying root water flow regulation. The present study showed that processes like phosphorylation/dephosphorylation and protein synthesis participate in regulating water transport in aspen seedling roots.

Previous studies using whole root systems and various metabolic inhibitors had shown that within one hour following the application of water flow inhibitors, there was a decrease in root water uptake, steady-state root water flow (Q_v, $m^3\ s^{-1}$) or water flux density (J_v, $m^3\ s^{-1}\ cm^{-3}$) (Maggio and Joly 1995, Tazawa et al. 2001, Kamaluddin and Zwiazek 2001). In aspen roots, naftenic acids decreased Q_v by 52-68% within 90 min following treatment (Kamaluddin and Zwiazek 2002), $HgCl_2$ decreased J_v by 50% within 30 min (Wan and Zwiazek 1999), and fluoride decreased Q_v by 73-76% after 90 min (Kamaluddin and Zwiazek 2003). The above studies suggest that water channel proteins in the root plasma membranes play a major role in mediating water transport in aspen roots. However, they provide little evidence for regulation mechanisms of aquaporin

function. Although there is no direct evidence, cell metabolism has been implied in the regulation of aquaporin activity (Zhang and Tyreman 1999, Kamaluddin and Zwiazek 2001, Kamaluddin and Zwiazek 2002). Following this line of investigation, the present study expanded the search for root water uptake regulation mechanisms in aspen seedling roots by examining some of the proposed mechanisms that could be used to mediate aquaporin activity (Johansson et al. 2000, Tyreman et al. 2002, Javot and Maurel 2002). Subsequently, Chapter 2 of the present study focused on protein phosphorylation and dephosphorylation events while Chapter 3 investigated the importance of protein synthesis with respect to the processes of water transport in roots.

The development of the composite model for water flow in roots suggested that, in unstressed plants, the flow of water through the apoplast is the predominant pathway with the contribution of water channel proteins being minor and required only for the fine regulation of root hydraulics (Steudle 2000, Zimmermann et al. 2000). However, studies using chemical inhibitors (Maggio and Joly 1995, Wan and Zwiazek 1999, Carvajal et al. 2000, Barrowclough et al. 2000) as well as the observations that roots can quickly alter L_p in response to different stresses (Steudle and Peterson 1998, Clarkson et al. 2000) suggest that the cell-to-cell pathway may play a more significant role in overall root water uptake than was previously thought. In the present study, the time-course and the extent of Q_v inhibition obtained with different chemical agents suggests the involvement of water channel proteins in root water flow. These results provide additional support to earlier observations concerning the pivotal role of aquaporins in root water relations of aspen (Wan and Zwiazek 1999, Kamaluddin and Zwiazek 2002).

The present study also suggests that root respiration is likely to have a secondary role in the regulation of root water uptake. The large decreases in Q_v obtained with different chemical inhibitors were not paralleled by similar decreases in root respiration (Chapter 2, Chapter 3). Interestingly, okadaic acid-treated roots maintained higher root respiration rates when compared to controls, although Q_v decreased (Chapter 2). Therefore, in the present study, the decrease in Q_v was not due to the decrease in root respiration, which was previously suggested to be the principal factor regulating the function of aquaporins (Kamaluddin and Zwiazek 2001, Kamaluddin and Zwiazek 2002). Root respiration decreased in control and treated seedlings likely due to the filling of previously aerated air spaces in the roots, under the influence of externally applied pressure (Markhart and Smit 1990). While a contribution of root respiration, probably through the supply of ATP for phosphorylation, is possible, it is unlikely that metabolic inhibition caused the decreased water flow in inhibitor treated-roots (Wan and Zwiazek 1999). The chemical inhibitors used in the present study were specific for protein kinases and phosphatases (Chapter 2) and protein synthesis (Chapter 3). Therefore, the processes of phosphorylation and dephosphorylation (Chapter 2) and protein synthesis (Chapter 3) are likely among the regulatory mechanisms for root water transport in aspen seedlings. However, the mechanism of aquaporin regulation appears to be complex and may also include other processes like the involvement of plasma membrane ATPases (Chapter 2).

In the present study, when hydrostatic forces were stopped for at least 30 min by excising the shoots or by incubating the roots, subsequent Q_v determination yielded a continuous increase in root water flow for approximately one more hour (Chapter 2). In excised roots, after the pressure is initially applied, water fluxes can increase slowly,

sometimes for several hours until a steady state is reached (Markhart and Smit 1990). However, a high positive pressure could force water along the root through the apoplastic pathways in the cortex that are not followed when hydrostatic forces are induced by transpiration (Huang and Nobel 1994, Markhart and Smit 1990). According to Markhart and Smit (1990), root systems that are placed under pressure for several hours appear water-soaked and translucent, due to the filling of air-spaces with water. Infiltration of roots by pressurization increased the radial hydraulic conductivity in both barley and onion roots (Tazawa et al. 1997, Tazawa et al. 2001). As discussed by Tazawa et al. (2001), if the increase in radial hydraulic conductivity is due to the formation of apoplastic longitudinal pathways as suggested by Passioura (1988), the increment of water uptake should be insensitive to $HgCl_2$. However, in barley root, the increase in root radial hydraulic conductivity after infiltration was more sensitive to $HgCl_2$ than before infiltration, which points to an increase in the area of the plasma membrane available for water transport (Tazawa et al. 1997).

In the present experiment, roots appeared translucent after the initial pressurization and remained as such during the incubation period. Assuming that the air-spaces were filled with water during the pressurization, they likely remained in this state during the measurements. Therefore the observed continuous increase of Q_v over time must be due to a process other than water-filling of air spaces. Interestingly, continuous increase of Q_v over time was observed for phosphorylation and dephosphorylation inhibitors but not for cycloheximide, a protein synthesis inhibitor. Therefore, the present study points to a possible protein involvement in the increased Q_v over time. Johansson et al. (1996) showed that aquaporin PM28A can be phosphorylated in response to increased apoplastic

water potential. By applying external pressure to previously detopped roots, the hydrostatic force that drove the water movement was increased by 0.3 MPa. Therefore, it is possible that positive external pressure applied to roots and greater than the initial apoplastic water potential in the intact plant, could result in the activation of certain aquaporins and increase the water flow over time. Phosphorylation and dephosphorylation inhibitors likely affected aquaporin activity while cycloheximide affected the amount of aquaporin protein in the plasma membrane. Therefore, the observed increase in Q_v could be due to modifications in the activity of proteins stimulated by the activation of water transport.

The present work showed that phosphorylation and dephosphorylation, and protein synthesis events may be involved in the rapid regulation of water uptake by aspen roots. The study also suggests a possible direct link between the activity of ATPases and the activity of aquaporins in the root plasma membranes. Inhibition of H^+-ATPase could result in local cytosolic acidification which can block the aquaporins (Fleurat-Lessard et al. 1997). The results of the present study are consistent with previous observations and complement the experiments with mercurials and other chemical inhibitors on aquaporin activity. The results suggest that in hydroponically-grown aspen seedlings, most of the water flux in roots is mediated by water channel proteins.

The contribution of water channel proteins to root water uptake is also important in maintaining water balance of the whole seedlings (Chapter 3). In intact seedlings, it is plausible that both hydraulic and chemical signals act together to bring about stomatal closure and preservation of the water within the plant (Maggio and Joly 1995). In intact plants, the water flow through the plasmodesmata (symplastic pathway) could also play

an important role (Zhang and Tyreman 1991). However, the present study did not assess this role. The transport of substances through the plasmodesmata also depends on phosphorylation and dephosphorylation events, and is mediated by proteins (Blackman and Overall 2001). Subsequently, the chemical inhibitors used in the present study, could also affect symplastic transport of water. On the other hand, Fricke (2000) showed that plasmodesmata close in response to pressures greater than 0.2 MPa. Most of the experiments in this study involved the measurements of roots pressurized at 0.3 MPa. Therefore, the plasmodesmata did not likely contribute significantly to water transport in the present study.

Similarly, the contribution of apoplastic transport to water flow regulation did not likely play a major role in the present study. The short time in which the inhibitory effects were observed suggests that anatomical changes were unlikely in the apoplast, since these changes take longer time to develop. However, changes in pH have been shown to modify the permeability of cell walls (Meychic and Yermakov 2001), thus modifying the contribution of apoplastic water flow. So far, there have been no studies examining the influence of different water flow inhibitors on the permeability of the apoplast within a root. Apoplastic barriers that develop over time could replace the need for regulation of aquaporin activity, but these barriers are usually irreversible (Tyreman et al. 2002).

The seedlings used in the present study were grown hydroponically under relatively unstressed conditions. However, one can argue that hydroponically-grown plants may suffer from mild hypoxia (Morard and Silvestre 1996). In the present study, to avoid hypoxic conditions solutions were aerated. Additionally, seedling appearance was

monitored over time to detect possible hypoxic responses and no symptoms of hypoxia (leaf chlorosis, leaf epinasty, decreased root elongation) were observed.

4.2. Technical problems

Some of the inhibitors used in this study were very expensive (e.g. H-7, staurosporine, okadaic acid, and W-7). Therefore, inhibitor solutions had to be reused for up to one week to ensure a reasonable number of replicates. However, the same was also done for the control solutions.

During that time, the solutions were kept at room temperature, which could have had some effect on their stability. Also, plant roots can modify their environment by ion exchange. All these factors could have influenced the measurements (Chapter 2). However, for the inhibitor treated-roots, the trend of Q_v obtained was similar for all the replicates. Therefore, potential change in the chemistry of treatment solutions was not likely a major factor in the present study.

4.3. Suggestions for future research

It is not clear from the present study whether phosphorylation and dephosphorylation events influence the aquaporin protein *in situ*, once inserted in the plasma membrane (aquaporin activity), or the synthesis and the insertion of water channel proteins into the membranes (aquaporin abundance). Further experiments are needed to clarify these aspects.

In the present study, the inhibition of Q_v obtained for Na_3VO_4, $Na_3PO_4x12H_2O$, and KF-treated roots was observed within 5 min. The same short time to Q_v inhibition was also observed for $HgCl_2$-treated roots (Wan and Zwiazek 1999). The timing of this inhibition was probably too short to allow for transport of the chemical inhibitor deep inside the root. Therefore, as suggested by Gaspar et al. (2001), the epidermal cell layer may have an essential role in controlling the water transfer in roots. Future experiments should reveal the roles of different tissues in controlling the water movement.

As suggested by the inhibition of root water uptake by a K^+ channel inhibitor (Tazawa et al. 2001), water fluxes in roots appear to be correlated with K^+ fluxes. According to Baiges et al. (2002), a regulatory scenario at low cell turgor, might involve an inactivated Ca^{2+} influx-channel or an activated Ca^{2+}-ATPase that would keep calcium outside the cytoplasm. This would inhibit the Ca^{2+}-dependent protein kinase and reduce the phosphorylation and the activity of PM28A aquaporin (Baiges et al. 2002). The present study points to a relation between the plasma membrane ATPases and water transport across roots (Chapter 2), as was also suggested by other studies (Fleurat-Lessard et al. 1997, Shiratake et al. 2001). Therefore, membrane water transport could also depend on nutrient transport, which is energy dependant (Sakano et al. 1992). However, more studies are needed to clarify the link between ATPases and aquaporin function in the plasma membranes.

The pH of the root medium plays an important role for extracting and transporting nutrients by roots. However, little has been done to explain pH effects on water transport in roots. Differences in pH have been shown to affect the water transport capacity of some mammalian aquaporins (Németh-Cahalan and Hall 2000, Zeuthen and Klaerke,

1999). Low pH can also have an influence on plant aquaporins (Gerbeau et al. 2002) and on water transport properties in roots (Gunsé et al. 1997). In the present study, the high pH of the Na_3VO_4 solution (Chapter 2) could have also affected the water transport. More studies are required to address the influence of pH on root water uptake.

In the present study, the incubation of roots in treatment solutions significantly increased subsequent Q_v values over time, with the exception of cycloheximide treatment. As previously suggested, aquaporins could be involved in this response possibly due to externally applied pressure. However, more experiments are needed to study the influence of pressure on the aquaporin activity.

The present study did not address the contribution of water flow through the apoplast and plasmodesmata to the total root water transport. In intact plants, particularly those exposed to environmental stresses, water transport through the apoplast and plasmodesmata could have a significant contribution to the total root water transport (Zhang and Tyreman 1991, Steudle and Peterson 1998). The chemical inhibitors applied in the present study could also have affected symplastic and apoplastic water transport. Future studies should reveal the contribution of different pathways to the total root water transport and their regulation under stress conditions.

Environmental stresses are known to affect root water flow. In order to determine how plants adapt to changes in their environment, one has to know how plant water relations are regulated. Therefore, the influence of different chemical inhibitors should also be extended to stress conditions.

References

Baiges, I., Schaffner, A.R., Affenzeller, M.J., and Mas, A. 2002. Plant aquaporins. Physiol. Plant. 115: 175-182.

Barrowclough, D.E., Peterson, C.A., and Steudle, E. 2000. Radial conductivity along developing onion roots. J. Exp. Bot. 344: 547-557.

Blackman, L.M., and Overall, R.L. 2001. Structure and function of plasmodesmata. Aust. J. Plant Physiol. 28: 709-727.

Carvajal, M., Cerdá, A., and Martinez, V. 2000. Does calcium ameliorate the negative effect of NaCl on melon root water transport by regulating aquaporin activity? New Phytol. 145: 439-447.

Clarkson, D.T., Carvajal, M., Henzler, T., Waterhouse, R.N., Smyth, A.J., Cooke, D.T., and Steudle, E. 2000. Root hydraulic conductance: diurnal aquaporin expression and the effects of nutrient stress. J. Exp. Bot. 51(342): 61-70.

Fleurat-Lessard, P., Frangne, N., Maeshima, M., Ratajczak, R., Bonnemain, J.-L., and Martinoia, E. 1997. Increased expression of vacuolar aquaporin and H^+-ATPase related to motor cell function in *Mimosa pudica* L. Plant Physiol. 114: 837-834.

Fricke, W. 2000. Water movement between epidermal cells of barley leaves - a symplastic connection? Plant Cell Environ. 23: 991-997.

Gaspar, M., Sissoëff, I., Bousser, A., Roche, O., Mahé, A., and Hoarau, J. 2001. Transient variations of water transfer induced by $HgCl_2$ in excised roots of young maize plants: new data on the inhibition process. Aust. J. Plant Physiol. 28: 1175-1186.

Gerbeau, P., Amodeo, G., Hnezler, T., Santoni, V., Ripoche, P. and Maurel, C. 2002. The water permeability of *Arabidopsis* plasma membrane is regulated by divalent cations and pH. Plant J. 30(1): 71-81.

Gunsé, B., Poschenrieder, C., and Barceló, J. 1997. Water transport properties of roots and root cortical cells in proton- and Al-sensitive maize varieties. Plant Physiol. 113: 595-602.

Huang, B., and Nobel, P.S. 1994. Root hydraulic conductivity and its components, with emphasis on desert succulents. Agron. J. 86: 767-774.

Javot, H., and Maurel, C. 2002. The role of aquaporins in root water uptake. Ann. Bot. 90: 301-313.

Johansson, I., Karlsson, M., Johansson, U., Larsson, C., and Kjelbom, P. 2000. The role of aquaporins in cellular and whole plant water balance. Biochim. Biophys. Acta 1465: 324-342.

Johansson, I., Larsson, C., Ek, B., and Kjellbom, P. 1996.The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^{2+} and apoplastic water potential. The Plant Cell 8: 1181-1191.

Kamaluddin, M., and Zwiazek, J.J. 2001. Metabolic inhibition of root water flow in red-osier dogwood (*Cornus stolonifera*) seedlings. J. Exp. Bot. 52 (357): 739-745.

Kamaluddin, M., and Zwiazek, J.J. 2002. Naphtenic acid inhibits root water transport, gas exchange and leaf growth in aspen (*Populus tremuloides*) seedlings. Tree Physiol. 22: 1265-1270.

Kamaluddin, M., and Zwiazek, J.J. 2003. Inhibition of root water transport by fluoride affects leaf expansion and gas exchange in aspen (*Populus tremuloides* Michx.) seedlings. Physiol. Plant. (in press).

Maggio, A., and Joly, R.J. 1995. Effects of mercuric chloride on the conductivity of tomato root system. Plant Physiol. 109: 331-335.

Markhart, A.H., and Smit, B. 1990. Measurement of root hydraulic conductance. HortSci. 25: 282-287.

Meychik, N.R., and Yermakov, I.P. 2001. Swelling of root cell walls as an indicator of their functional state. Biochem. 66(2): 178-187.

Morand, P., and Silvestre, J. 1996. Plant injury due to oxygen deficiency in the root environment of soilless culture: a review. Plant Soil 184: 243-254.

Németh-Cahalan, K., and Hall, J.K. 2000. pH and calcium regulate the water permeability of aquaporin 0. J. Biol. Chem. 275(10): 6777-6782.

Passioura, J.B. 1988. Water transport in and to roots. Ann. Rev. Plant. Physiol. Plant Mol. Biol. 34: 245-265.

Sakano, K., Yazaki, Y., and Mimura, T. 1992. Cytoplasmic acidificatio induced by inorganic phosphate uptake in suspension cultured *Cantharanthus roseus* cells. Plant Physiol. 99: 672-680.

Shiratake, K., Goto, H., Maeshima, M., and Yamaki, S. 2001. Fluctuations in the protein levels of H^+-pumps and water channels of the tonoplst and plasma membrane during grape berry development. J. Japan. Soc. Hort. Sci. 70(3): 287-293.

Steudle, E. 2000. Water uptake by the plant roots: an integration of views. Plant Soil 226: 45-56.

Steudle, E., and Peterson, C.A. 1998.How does water get through roots? J. Exp. Bot. 49(322): 775-778.

Tazawa, M., and Okazaki, Y. 1997. Water channels does not limit evaporation of water from plant cells. J. Plant Res. 110: 317-320.

Tazawa, M., Sutou, E., and Shibasaka, M. 2001. Onion root water transport sensitive to water channel and K+ channel inhibitor. Plant Cell Physiol. 42(1): 28-36.

Tyreman, S.D., Niemietz, C.M., and Bramley, H. 2002. Plant aquaporins: multifunctional water and solute channels with expanding roles. Plant Cell Environ. 25: 173-194.

Wan, X., and Zwiazek, J.J. 1999. Mercuric chloride effects on root water transport in aspen seedlings. Plant Physiol. 121: 939-946.

Zeuthen, T., and Klaerke, D.A. 1999. Transport of water and glycerol in aquaporin 3 is gated by H^+. J. Biol. Chem. 274(31): 21631-21636.

Zhang, W.H., and Tyreman, S.D. 1991. Effect of low O_2 concentration on azide on hydraulic conductivity and osmotic volume of the cortical cells of wheat roots. Aust. J. Plant Physiol. 18: 603-613.

Zhang, W.H., and Tyreman, S.D. 1999. Inhibition of water channels by $HgCl_2$ in intact wheat root cells. Plant Physiol. 120: 849-857.

Zimmermann, H.M., Hartmann, K., Schreiber, L., and Steudle, E. 2000. Chemical composition of apoplastic barriers in relation to radial hydraulic conductivity of corn roots (*Zea mays* L.). Planta 210: 302-311.

Appendix A

ANOVA tables for phosphorylation/ dephosphorylation data (Chapter 2)

1. Steady-state root water flow (Q_v)

H-7

Effect	Num DF	Den DF	F Value	Pr > F
trt	1	18.7	0.26	0.6154
Day	3	12.5	0.51	0.6830
period	11	66.9	5.67	<.0001
trt*period	11	66.9	1.18	0.3172

Staurosporine

Effect	Num DF	Den DF	F Value	Pr > F
trt	1	14.1	1.65	0.2202
Day	2	5.83	7.34	0.0256
period	17	42.6	7.50	<.0001
trt*period	17	42.6	0.69	0.7965

6-DMAP

Effect	Num DF	Den DF	F Value	Pr > F
trt	2	15.3	6.91	0.0073
Day	1	13.3	1.53	0.2382
period	17	69.6	6.90	<.0001
trt*period	34	94.3	2.26	0.0011

KF and Na_3VO_4

Effect	Num DF	Den DF	F Value	Pr > F
trt	5	46	53.10	<.0001
period	11	506	153.85	<.0001
trt*period	55	506	8.65	<.0001

* no degrees of freedom for Day

OA- 0.2 μM

Effect	Num DF	Den DF	F Value	Pr > F
trt	1	5.35	0.38	0.5635
Day	5	4.8	0.23	0.9326
period	23	1	80.71	0.0877
trt*period	23	1	1.84	0.5311

OA- 0.2 μM + KF

Effect	Num DF	Den DF	F Value	Pr > F
trt	1	7	0.26	0.6238
Day	3	7	1.46	0.3052
period	11	110	6.48	<.0001
trt*period	11	110	1.16	0.3268

OA- 0.1 μM

Effect	Num DF	Den DF	F Value	Pr > F
trt	1	13.2	16.09	0.0014
Day	5	5.69	3.72	0.0752
period	29	36.3	19.65	<.0001
trt*period	29	36.3	1.82	0.0434

W-7

Effect	Num DF	Den DF	F Value	Pr > F
trt	2	17	2.13	0.1492
Day	3	32	4.89	0.0065
period	23	58.6	15.46	<.0001
trt*period	46	85	1.23	0.2042

ANOVA tables for phosphorylation/ dephosphorylation data (Chapter 2)

2. Root O_2 uptake

6-DMAP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.01209546	0.00403182	0.56	0.6579
Error	8	0.05788924	0.00723615		
Corrected Total	11	0.06998470			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	1	0.00706390	0.00706390	0.98	0.3521
Day	1	0.00473933	0.00473933	0.65	0.4418
trt*Day	1	0.00194770	0.00194770	0.27	0.6179

KF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.08638556	0.01234079	1.80	0.1933
Error	10	0.06870650	0.00687065		
Corrected Total	17	0.15509206			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	1	0.00089567	0.00089567	0.13	0.7256
Day	3	0.06922459	0.02307486	3.36	0.0634
trt*Day	3	0.01612025	0.00537342	0.78	0.5306

Na_3VO_4

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.37298008	0.05328287	3.57	0.0476
Error	8	0.11951359	0.01493920		
Corrected Total	15	0.49249367			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	1	0.02250643	0.02250643	1.51	0.2546
Day	3	0.07854410	0.02618137	1.75	0.2337
trt*Day	3	0.27192955	0.09064318	6.07	0.0186

OA

Effect	Num DF	Den DF	F Value	Pr > F
trt	1	6.38	7.22	0.0341
Day	5	6.55	0.36	0.8576
period	2	19.5	25.22	<.0001
trt*period	2	19.5	3.43	0.0427

ANOVA tables for phosphorylation/ dephosphorylation data (Chapter 2)

3. Acid phosphatase assay

KF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1.63309347	0.23329907	99.38	<.0001
Error	16	0.03756223	0.00234764		
Corrected Total	23	1.67065570			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	3	1.57316922	0.52438974	223.37	<.0001
Day	1	0.04343151	0.04343151	18.50	0.0005
Day*trt	3	0.01649275	0.00549758	2.34	0.1118

Na_3VO_4

pH unadjusted

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1.76754627	0.25250661	179.19	<.0001
Error	16	0.02254619	0.00140914		
Corrected Total	23	1.79009246			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	3	1.49855466	0.49951822	354.49	<.0001
Day	1	0.05272117	0.05272117	37.41	<.0001
Day*trt	3	0.21627045	0.07209015	51.16	<.0001

pH adjusted to 5

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	7	1.32605099	0.18943586	44.49	<.0001
Error	16	0.06813009	0.00425813		
Corrected Total	23	1.39418109			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	3	1.30877966	0.43625989	102.45	<.0001
Day	1	0.01163171	0.01163171	2.73	0.1179
Day*trt	3	0.00563962	0.00187987	0.44	0.7265

ANOVA tables for phosphorylation/ dephosphorylation data (Chapter 2)

OA *in vitro* phosphatase assay

pH 5

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.00244973	0.00034996	0.86	0.5574
Error	16	0.00651652	0.00040728		
Corrected Total	23	0.00896625			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	3	0.00107638	0.00035879	0.88	0.4718
group	1	0.00065519	0.00065519	1.61	0.2228
group*trt	3	0.00071816	0.00023939	0.59	0.6318

pH 7.5

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.01371413	0.00195916	3.23	0.0248
Error	16	0.00971849	0.00060741		
Corrected Total	23	0.02343262			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	3	0.00475163	0.00158388	2.61	0.0875
group	1	0.00607549	0.00607549	10.00	0.0060
group*trt	3	0.00288701	0.00096234	1.58	0.2322

OA- *in vivo* phosphatase assay

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	0.00002276	0.00000379	1.04	0.4921
Error	5	0.00001819	0.00000364		
Corrected Total	11	0.00004096			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	1	0.00000762	0.00000762	2.09	0.2076
Day	5	0.00001514	0.00000303	0.83	0.5773

* no degrees of freedom for trt*Day

Appendix B

ANOVA tables for cycloheximide data (Chapter 3)

Steady-state root water flow (Q_v)

Effect	Num DF	Den DF	F Value	Pr > F
trt	3	18.1	19.64	<.0001
Day	6	11.1	3.35	0.0393
period	17	113	24.56	<.0001
trt*period	51	171	2.39	<.0001

Steady-state root water flow (Q_v) and root respiration

Effect	Num DF	Den DF	F Value	Pr > F
trt	3	18.1	19.64	<.0001
Day	6	11.1	3.35	0.0393
period	17	113	24.56	<.0001
trt*period	51	171	2.39	<.0001

Root respiration

Effect	Num DF	Den DF	F Value	Pr > F
trt	1	25	0.38	0.5414
Day	5	25	1.29	0.3006
period	2	25	300.30	<.0001
trt*period	2	25	4.42	0.0226

Stomatal conductance

Effect	Num DF	Den DF	F Value	Pr > F
trt	1	13	0.94	0.3508
Day	2	7.78	2.04	0.1942
period	4	21.6	20.47	<.0001
trt*period	4	21.6	9.45	0.0001

In vitro acid phosphatase assay

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.03563441	0.00509063	4.28	0.0076
Error	16	0.01902832	0.00118927		
Corrected Total	23	0.05466273			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	3	0.02873711	0.00957904	8.05	0.0017
group	1	0.00029990	0.00029990	0.25	0.6224
group*trt	3	0.00659740	0.00219913	1.85	0.1790

Leaf water xylem pressure (Ψ_w)

Effect	Num DF	Den DF	F Value	Pr > F
trt	1	6.4	12.64	0.0108
Day	1	9.48	2.87	0.1226
period	2	12	20.50	0.0001
trt*period	2	12	11.58	0.0016

ANOVA tables for cycloheximide data (Chapter 3)

Root hydraulic conductivity (L_p)

0 min

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	1.400833E-22	1.400833E-22	0.01	0.9374
Error	10	2.157108E-19	2.157108E-20		
Corrected Total	11	2.158509E-19			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	1	1.400833E-22	1.400833E-22	0.01	0.9374

* no degrees of freedom for Day

60 min

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1.107995E-18	1.582849E-19	18.71	0.0066
Error	4	3.384077E-20	8.460193E-21		
Corrected Total	11	1.141835E-18			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	1	9.542996E-19	9.542996E-19	112.80	0.0004
Day	3	1.080536E-19	3.601787E-20	4.26	0.0977
trt*Day	3	1.404633E-19	4.682109E-20	5.53	0.0659

120 min

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1.100096E-18	2.200192E-19	25.28	0.0006
Error	6	5.221263E-20	8.702105E-21		
Corrected Total	11	1.152309E-18			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	1	9.337572E-19	9.337572E-19	107.30	<.0001
Day	2	1.117675E-19	5.588377E-20	6.42	0.0323
trt*Day	2	5.457127E-20	2.728563E-20	3.14	0.1169

Apoplastic water flow ratio

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.00722131	0.00180533	3.59	0.0349
Error	13	0.00653325	0.00050256		
Corrected Total	17	0.01375456			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	1	0.00425861	0.00425861	8.47	0.0121
Day	3	0.00295241	0.00098414	1.96	0.1702